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Molecular Phylogenetics of Floridian Boletes

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by

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DEDICATION

This work is dedicated to those who have supported me, helped me, and collected mushrooms with me: Gretchen Farid, Mostafa Farid, Bijan Farid, Arman Farid, Rose E. O'Donovan, John O'Donovan, Alida O'Donovan, Dorian L. Cardenas, Sean O'Donovan, Jon O'Donovan, Shawna O'Donovan. To my friends: Richard W. McKenzie, Garrett Speed, Lori Collins, Travis Doering, Christian K. Marr, Abbey Bridges, Steven Heid, Vincent Meijer, Myriam van Walsum, Agí Muszbek, Stu McKenzie, Ambrose Kenny-Smith, Cook Craig, Joey Walker, Lucas Skinner, Michael Cavanagh, and Eric Moore, Bobby Scharping, Madison Davis, Christina Moss, Vickie Frazier, Robert Madden, and Richard Nguyen.

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ABSTRACT

The boletes are macrofungi which have undergone extensive taxonomic revisions since the advent of molecular tools. To further our understanding of the boletes in peninsular Florida, we sequenced two common Floridian boletes, and analyzed them with molecular phylogenetic tools. *Boletus rubricitrinus*, a common Florida bolete often found in lawns under *Quercus*, and likely has a distribution that extends to Texas. Based on ITS and LSU sequences and morphological studies, this species belongs in the genus *Pulchroboletus*. As the holotype is in poor condition, an epitype is established here. A thorough description of macroscopic and microscopic features is also provided for the species. Fungi in the genus *Phylloporus* are lamellate boletes that occur worldwide, but primarily in the tropics. *Phylloporus boletinoides* is a species which was described from Florida, and is found growing near *Pinus* spp. Based on ITS, LSU, and RPB1 sequences, we establish the novel genus *Pseudophylloporus*, which is allied to *Bothia* and *Solioccasus*. Morphological data are also provided from our collections, and one from Belize. Based on molecular data and a review of bolete literature, the delimitation of this genus suggests that there are three distinct lineages of boletes that have a lamellate hymenium in the Boletaceae. These molecular and morphological data will be useful to further improve our understanding of bolete taxonomy.

CHAPTER ONE: INTRODUCTION

Fungi are a diverse kingdom that encompasses both unicellular and multicellular organisms. Fungi were previously considered to be primitive plants, but modern DNA phylogenetics place fungi closer to animals, in a group called Opisthokonta (Melinda et al. 2003). Many of the basal Fungi are flagellate and unicellular, a trait shared with most other Opsithokonts (Melinda et al. 2003).

Fungi encompass a variety of ecological functions. One primary role is the decomposer, also referred to as the saprobe, which recycles decayed plant matter back into the ecosystem (Sánchez 2009). Many Fungi are also form symbioses with other organisms. Lichen-forming fungi are the symbiosis of fungi and a photobiont. This nutritional mode accounts for 20% of described fungal species, and is a trait which has arisen 20–30 times in the Fungi (Lücking 2016). Many fungi are farmed by a diversity of insects, providing nutrition in exchange for habitation (Mueller et al. 2005). There are also several groupings of fungi that are known as mycorrhizal fungi (Hibbett et al., 2000). Mycorrhizal fungi form symbiotic partnerships with plant roots (through the rhizoids in liverworts; see Field et al. 2016), playing an essential role in the vitality of plants by providing nitrogen and phosophorus in exchange for carbon in the form of sugars (Landeweert et al. 2003, Perez-Moreno & Read 2009, Smith & Smith 2011). Mycorrhizal fungi also provide plants with resistance to drought (Augé 2001), salinity (Evelin et al. 2009), heavy metal toxicity (Tam 1995), and acidic soils (Malloch et al. 1980, Clark et al. 1999). Most plants, about 80%, associate with mycorrhizal fungi (Wang & Qui, 2006). There are two general types of mycorrhizal fungi, arbuscular mycorrhizal and ectomycorrhizal fungi. Arbuscular mycorrhizal fungi are found exclusively in the Glomeromycota (Smith & Smith 2011). Glomeromycota form structures called arbuscules which penetrate the cells of plant roots to maximize nutrient exchange (Smith & Smith 2011). Ectomycorrhizal fungi sheath

the outer cortex of plant roots with hyphae (Blasius et al. 1986). Ectomycorrhizal fungi have independently arisen multiple times in the Zygomycota, Ascomycota, and Basidiomycota (Tedersoo et al., 2010).

1.1 The boletes

One distinctive group of the Basidiomycota are the boletes, which reside in the order Boletales. A typical bolete has a soft spongy cap (pileus), central stem (stipe), and a tubulose hymenophore (Fig. 1.1). Boletes typically make up a large portion of the visible mycobiota during the rainy seasons (Hongo 1984), and are consumed by many forest animals (Bruns 1984). Boletes are a source of food for humans, with some species among the most prized choice edibles (Hall et al. 1998, Kuo 2007, Sanmee et al. 2010). Recent research has suggested that boletes may have even been a part of the human diet in the Magdalenian period, about 18,000 to 12,000 years ago (Power et al. 2015). Some boletes are poisonous, such as *Rubroboletus satanus* (Lenz) Kuan Zhao & Zhu L. Yang, which contains bolesatine, a toxic glycoprotein that inhibits protein synthesis in human kidneys (Kretz et al. 1991). Boletes are efficient at heavy metal uptake from the environment (Malinowska et al. 2004), a promising avenue for environmental bioremediation (Elekes & Busuioc 2011). Pigments may be extracted from boletes to make dyes for fabrics (Bessette and Bessette, 2001), an environmentally friendly alternative to typical commercial dyes (Durán et al. 2002, Velíšek & Cejpek 2011).

1.2 The diversity of the Boletales

The Boletales form a monophyletic clade within the Agaricomycetideae, alongside the Atheliales and Agaricales (Hibbett et al 2007). Molecular dating suggests the Boletales diverged from the Agaricales 189 million years ago (Feng et al. 2012). The Boletales are a diverse group of fungi with many different basidiocarp forms, including resupinate, gasteroid, secotioid, hypogenous, lamellate, and tubulose. The earliest extant tubulose members of the Boletales evolved in the Suillineae (Binder & Hibbett 2006). Suillineae are ecologically and moprhologically diverse; *Suillus* Gray are tubulose fungi that are important ectomycorrhizal partners of Pinaceae host species (Binder & Hibbett 2006), while *Chroogomphus* (Singer) O.K. Mill. are lamellate fungi that are exclusively parasitic on boletes (Olsson et al. 2000). The Sclerodermatineae contains gasteroid genera such as *Scleroderma* Pers. and *Calostoma* Desv., as well as

saprobic genera *Phlebopus* (R. Heim) Singer (Binder & Hibbett 2006). One species in the Sclerodermatineae, *Boletinellus merulioides* (Schwein.) Murrill, is inhabited by leafcurl ash aphids (*Meliarhizophagus fraxinifolii*), which are parasitic on *Fraxinus*, and excrete nutrient-rich honeydew which is then absorbed by *B. merulioides* (Nuhn 2016:1-2). Most boletoid fungi are found in the Boletaceae (Nuhn et al. 2013, Wu et al. 2014, Nuhn 2016, Wu et al. 2016).

Most boletes are ectomycorrhizal and tend to have very specific host preferences (Kropp & Trappe 1982, Molina & Trappe 1982; Watling & See 1995). Switch in host preference has been shown to drive diversification in the boletes (Egger & Hibbett 2004, Den Bakker et al. 2008, Halling et al. 2008. Feng et al. 2012). For instance, a study by Sato et al. (2016) demonstrated that ancestral lineages of *Afroboletus* Pegler & T.W.K. Young and *Strobilomyces* Berk. experienced extensive speciation as it switched host lineages from African plants in the subfamilies Caesalpiniodeae and Monotoideae to tropical Asian plants in the Dipterocarpoideae, where it radiated to the temperate Eurasian plants in the Fagaceae and Pinaceae, and Australian plants in the Nothofagaceae.

1.3 Rationale

Florida has received attention from relatively few mycologists regarding its mycological diversity. William Alphonso Murrill was an early mycologist who studied Florida's fleshy fungi diversity, primarily from 1927 –1957. Murrill made several collecting trips early in his career as a mycologist at the New York Botanical Gardens, and after an illness caused him to lose his job, he decided to move to Florida, partially because of the diversity of fleshy fungi present here (Weber 1961). He described over 1,400 species and 80 genera of fleshy fungi in his career, many of these from Florida, until his death in 1957 (Halling 1986). Another mycologist who contributed to the understanding of bolete diversity of Florida was Rolf Singer. His monograph, the Boletoideae of Florida (Singer 1945, 1945a, 1947), treated 65 species from Florida.

The focus of this study was to use a molecular framework to analyze two species of boletes that are common in Florida, *Boletus rubricitrinus* Murrill and *Phylloporus boletinoides* A.H. Smith & Thiers. The Boletaceae has been subject to many taxonomic rearrangements, often due to homoplasious or plesiomorphic fruiting body forms, colors, discoloration of basidiomes, misapplied European names, and

lack of rigorous microscopic studies (Hibbett et al. 1997, Binder & Bresinsky 2002, Binder et al. 2005, Binder & Hibbett, 2006, Nuhn et al. 2013, Wu et al. 2014, 2016). Singer (1986) treated 25 genera in the Boletaeae (Singer 1986), which has inflated to over 60 genera (Wu et al. 2014, Wu et al. 2016). Many concepts of genera have been heavily revised and narrowed due to their extensive polyphyly.

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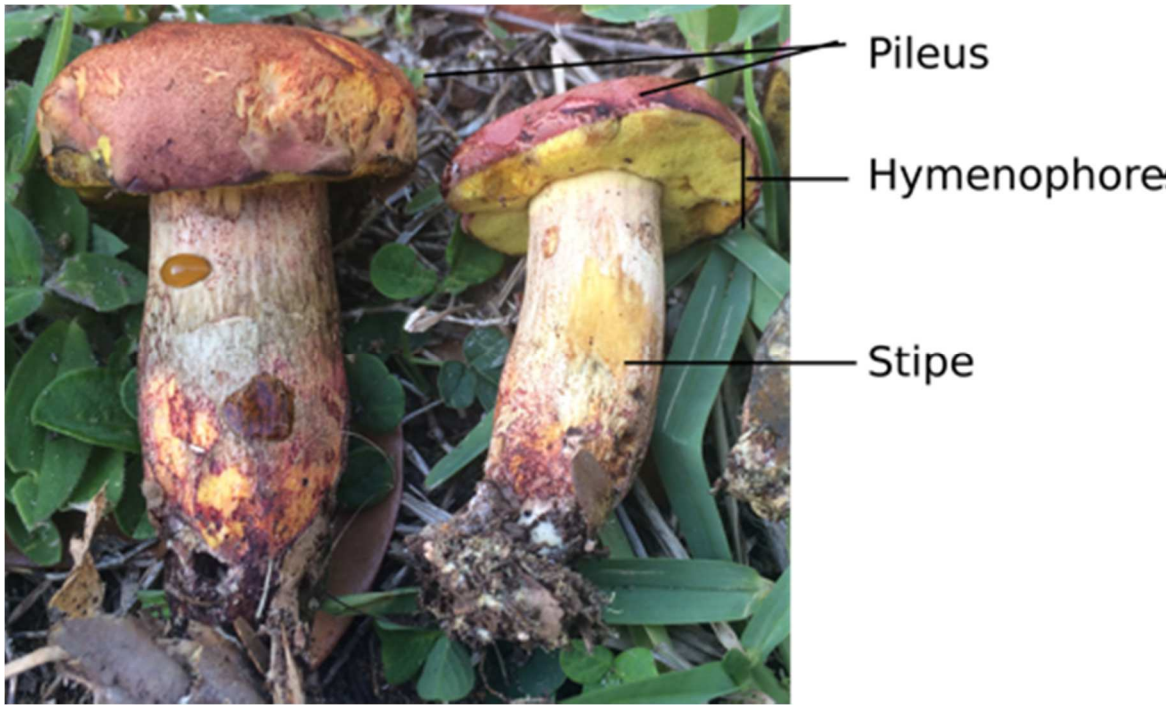

Fig. 1.1. The anatomy of a typical bolete. Photo by A. Farid.

CHAPTER TWO: *BOLETUS RUBRICITRINUS* BELONGS IN THE GENUS *PULCHROBOLETUS* (BOLETACEAE)

Note to Reader. Portions of this chapter have been accepted for publication (Farid et al. 2017) and are used with permission from the publisher.

2.1 Introduction

The boletes are a polyphyletic assemblage of macrofungi in the *Boletales* which are defined by stipito-pileate basidiomes with tubulose hymenophores. They were first placed in *Boletus* L. (Linnaeus 1753: 1176) and *Boletaceae* (Chevallier 1826: 248), obsolete concepts which both included polypores. The order *Boletales* was later introduced by Gilbert (1931) to exclusively include boletes. Molecular phylogenetic tools (Martin et al. 2011) have expanded the *Boletales* to include agaricoid, resupinate, and gasteroid fungi (Bruns et al. 1989; Hibbett et al. 1997. Binder & Bresinsky 2002b; Binder & Hibbett 2006). There are over 1300 species in the *Boletales*, comprised of 17 families and about 100 genera (Kirk et al. 2008). *Boletaceae* sensu stricto now contains about 70 genera and approx. 800 species (Bresinsky et al. 1999, Binder & Bresinsky 2002a; Binder & Hibbett 2006; Drehmel et al. 2008; Desjardin et al. 2009, Orihara et al. 2010, Li et al. 2011, Nuhn et al. 2013, Gelardi et al. 2014, Wu et al. 2014).

There are approximately 300 species of *Boletus* sensu Kirk et al. (2008: 97), although the number is likely to change as more molecular data become available. *Boletus* sect. *Luridi* Fr. (1838: 417), the largest section in *Boletus* sensu Singer (1986: 778), contained 40 species, defined by small, discoloring pores with finely reticulated or furfuraceous stipes. Molecular investigations found *Boletus* sect. *Luridi* to be polyphyletic, resulting in the transfer of species to existing or

novel genera within *Boletaceae* (Takahashi et al. 2011, Vizzini 2014a, 2014b, 2014c, Vizzini et al. 2014, Zhao et al. 2014).

Boletus rubricitrinus (Murrill) Murrill is a bolete with a brick-colored pileus, a yellow stipe with red floccules/punctules concentrated at the base, and a usually acidic taste (Murrill 1940). It was described from a collection made on a lawn near *Quercus laurifolia* in Gainesville, Florida, USA and originally placed in *Ceriomyces* Battarra ex Murrill, nom. illeg. (Murrill 1940; see Donk 1958: 167 for interpretation of Battarra names). Singer placed *B. rubricitrinus* in *Boletus* sect. *Luridi* (Singer 1947, Singer 1986).

Boletus rubricitrinus has not yet been analyzedwith molecular tools. In this study, we aim to understand the taxonomic placement of *B. rubricitrinus* in the context of molecular DNA evidence. Also, since this species lacks modern rigorous morphological descriptions, we provide a more detailed microscopic description.

2.2 Materials and Methods

2.2.1 Sampling and identification

Specimens examined were collected in peninsular Florida between 2012–2017 and deposited at the University of South Florida Herbarium (USF).

Specimens were identified based on the protologue (Murrill 1940), Murrill's identification keys (Murrill 1972), and examination of the holotype.

2.2.2 Morphological studies

Macroscopic descriptions are based on detailed notes made from fresh basidiomes. Micromorphological features were observed from dried specimens using a compound microscope (AccuScope, Commack, NY, USA); distilled H_2O, 5% KOH, and Congo red were used to rehydrate and stain sections. Measurements were made at 1000× with a calibrated ocular

micrometer. Micrographs were taken with a Nikon D3200 camera. Basidiospore dimensions are reported as length by width, with each measurement reported as the minimum, the average minus the standard deviation, the average plus the standard deviation, and the maximum. Measurements are followed by the number of spores counted, and the average quotient Q, where Q = average length divided by average width.

2.2.3 DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was isolated from dried herbarium specimens (Tab. 1) using a modified CTAB extraction protocol (Doyle & Doyle 1987; Franck et al. 2012); the resulting DNA was diluted in 65 µl of a 10 mM Tris, 1 mM EDTA buffer. Universal primers ITS1/ITS4 were used to amplify ITS1, 5.8S rRNA, and ITS2 (White et al. 1990). The primer pair LR0R/LR7 (Vilgalys & Hester 1990) were used to amplify 28S rRNA (LSU). Amplification reactions were performed on a T3 Thermocycler (Biometra, Göttingen, Germany) with 20 µl volumes, using 1 unit ID*Proof*™ Taq Polymerase (Empire Genomics, Buffalo, NY, USA), 2 µl 10× Reaction Buffer, 3 mM $MgCl_2$, 120 ng of each primer, 250 µM dNTPs, and 1 µl of DNA. If amplification failed, serial dilutions were used for additional attempts. Amplification cycle parameters for the ITS region were as follows: 94 ºC for 3 minutes for initial denaturation, followed by 40 cycles of 94 ºC for 45 s, 51 ºC at 45 s for annealing, and an extension at 72 ºC for 90 s, with a final extension of 72 ºC for 5 minutes. Amplification cycle parameters for the LSU region were as follows: 95 ºC for 2 minutes for initial denaturation, followed by 40 cycles of 94 ºC for 45 s, 50 ºC at 70 s for annealing, and an extension at 72 ºC for 120 s, with a final extension of 72 ºC for 10 minutes. Samples were visualised in 0.9% agarose using TAE buffer and 1% ethidium bromide to ensure product of expected size was produced. Crude PCR product was purified and sequenced at the DNA Laboratory at Arizona State

University with a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) using the same PCR primers and an additional internal primer for LSU, LR5 (Vilgalys & Hester 1990).

2.2.4 Sequence alignment, dataset assembly, and phylogenetic analysis

Sequences obtained in this study were run using the BLASTn algorithm (Boratyn et al. 2013) to identify related sequences. These sequences were combined with sequences from the literature (Morris et al. 2008, Smith & Pfister 2009, Gelardi et al. 2014, Frank et al. 2017) for phylogenetic analysis (Tab. 1). Sequences were aligned for ITS and LSU using the Clustal W algorithm (Thompson et al. 1994) in MEGA7 (Kumar et al. 2016) with default parameters. Phylogenetic analyses were run for ITS and LSU separately, as well as a concatenated ITS/LSU dataset.

Phylogenetic hypotheses were constructed with Bayesian inference (BI) and Maximum parsimony (MP) methods. The best-fit substitution models for both corrected Akaike information criterion (AICc) and Bayesian information criterion (BIC) were determined by jModelTest 2.1.10 (Guindon & Gascuel 2003, Darriba et al. 2012). The BIC model provided for ITS, K80+G, was used for the BI analysis; the BIC model provided for LSU, TrNef+I, was used. BI was conducted with MrBayes version 3.2.6 (Ronquist et al. 2012) with four Markov chain Monte Carlo 10,000,000 generations, sampling trees every 1,000 generations, resulting in 10,001 trees; the first 25% were discarded as burn-in, and a majority rule consensus tree was computed to obtain estimates for Bayesian posterior probabilities (BPP). BPP equal to and above 0.50 were reported. The analysis was also run for both gene regions with the AICc model provided by jModelTest 2.1.10, and produced the same topology with similar BPP. MP analysis was conducted with PAUP* version 4.0a152 (Swofford 2002) with 1,000 bootstrap replicates (Felsenstein 1985) using a heuristic search; starting trees for branch-swapping were obtained by stepwise addition, and the tree-bisection-reconnection algorithm was used for branch swapping. Bootstrap supports (BS)

equal to or greater than 50% were reported. Bayesian consensus trees were visualised in FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/), with BPP displayed as node labels. Bayesian consensus trees were then exported as a scalable vector graphic and imported into Inkscape version 0.91 (http://www.inkscape.org) to re-annotate tip labels and add BS. Alignment and phylogenetic trees were uploaded to http://www.treebase.org/ (submission ID 21355).

2.3 Results

2.3.1 Molecular analysis

Four ITS sequences and one LSU sequence were obtained from four specimens of *Boletus rubricitrinus* selected for study. The final ITS dataset consisted of our four new sequences and 18 sequences from the literature. These 22 sequences corresponded to six known species, while three environmental sequences from the literature were unidentified members of *Boletaceae*. Both BI and MP produced the same topology. The four newly sequenced *Boletus rubricitrinus* samples clustered as a sister clade to *Pulchroboletus roseoalbidus* (Alessio, Galli & Littini) Gelardi, Vizzini & Simonini with 1.0 BPP and 99.15% BS (Fig. 1). The three environmental sequences formed a sister group to the *Pulchroboletus* clade, with 1.0 BPP and 99.642% BS: EU569236.1, identified as *Boletus* sp., with a voucher collected in Guerrero, Mexico, FM999554.1, an uncultured environmental sequence from Ohio, USA, and FJ480444.1, identified as *Boletus* sp., collected in Massachusetts, USA, with submission notes of the isolation source having a bright orange sclerotium.

The LSU dataset consisted of one new sequence and 12 sequences from the literature. These 13 sequences corresponded to the same six named species as the ITS tree. For LSU, both BI and MP produced a topology which is congruent with the ITS tree. *Boletus rubricitrinus* formed a

sister clade to four *Pulchroboletus roseoalbidus* samples, with 0.9812 BPP and 96.233% BS (Fig. 2). The combined LSU/ITS dataset topology was congruent with the ITS and LSU topologies (Fig. 3). *Boletus rubricitrinus* formed a sister clade to four *P. roseoalbidus* samples, with 1.0 BPP and 100% BS.

2.3.2 Taxonomy

Pulchroboletus rubricitrinus (Murrill) A. Farid & A.R. Franck, **comb. nov.** Figs. 4–8

(Mycobank MB 821474)

Basionym: *Ceriomyces rubricitrinus* Murrill, Bull. Torrey Bot. Club 67(1): 61 (1940).

≡ *Boletus rubricitrinus* (Murrill) Murrill, Bull. Torrey Bot. Club 67(1): 66 (1940).

Holotype. USA, Florida, Alachua Co., Gainesville, lawn near laurel oak [*Quercus laurifolia*], 2 July 1938, W.A. Murrill s.n. (FLAS F-17321).

Epitype (designated here, MycoBank MBT 378921). USA, Florida, Hillsborough Co., University of South Florida campus, along S side of sidewalk, N of Alumni Drive and S of Richard Beard garage, lawn, beneath *Quercus virginiana*, 10 June 2016, Arian Farid 335 (USF 288420). GenBank sequences MF193884 (ITS), MG026638 (LSU).

Examination of holotype

Dried basidiome. Pileus dark brown-olive, occasionally faintly maroon-testaceous in centre, smooth, glabrous. Tubes adnexed-decurrent with a tooth, not separable individually, dark brown, pore mouths subangular. Stipe striate, brownish with a tinge of maroon-red. Mould (*Aspergillus* sp.) damage present on the pileus and stipe of basidiome, and parts of hymenium.

Microscopic features. Basidiospores (12.9)13.4–16.0(18.5) × (3.7)4.3–6.3(6.8) µm (40 spores counted, Q = 2.8), straw-yellow in KOH and water, ellipsoidal to subellipsoidal, sometimes subfusiform, smooth, thin-walled, with a pronounced apiculus and rounded apex, and only rarely

with one, two, or three olive-colored oil droplets (these not lasting over time; Murrill's original protologue defines them as these droplets, which are seen in his drawing alongside the specimen).

Basidia 12.7–25.2 × 10.6–12.2 μm, clavate, subclavate, or cylindrical, smooth, thin-walled, hyaline, yellow-green oil droplets in water and KOH, without basal clamps; sterigmata 1–3 μm long; basidioles clavate to subclavate, size similar to basidia.

Cheilocystidia 19.6–37.5 × 8.4–12.1 μm, light brownish to hyaline in KOH, sometimes encrusted with yellow-green oil droplets, these very small, ventricose to capitulate, clavate, somewhat strangulated at times, apices subclavate to filiform, fusoid. Pleurocystidia shape and size similar to cheilocystidia.

Hymenophoral trama bilateral, boletoid, lateral strata somewhat gelatinised, elements 5–14 μm wide, mediostrata gelatinised, loosely arranged, yellow-brown, hyphae 5–14 μm wide.

The holotype material has sustained much mould damage over time. Although the above features found in the holotype match our other examined collections, other microscopic features could not be discerned through the mould, such as the pileipellis, context, and stipitipellis. Attempts to remove the contaminant mould were attempted, but not successful. Accordingly, we designate an epitype from our sequenced specimens of which we also have photographs.

Emended description

The description is based only on material examined which was also successfully sequenced: Farid 335, Franck 3114, 3473, 3594 (for details, see Appendix).

Fresh basidiome. Pileus 3–16 cm diameter, at first hemispherical to pulvinate, then becoming convex, then plane, firm when young, becoming soft and fleshy with age; margin involute when young, becoming expanded, uplifted, occasionally lobed, especially when young, occasionally

exceeding approx. 1 mm beyond tubes; cuticle somewhat greasy, smooth, occasionally pitted at maturity, glabrous, pink, testaceous, blood-red, with testaceous, vinaceous, or maroon punctules.

Tubes yellow, rounded when young, becoming subangular to angular when mature, adnate, then becoming decurrent with a tooth, tubes separable individually, 0.5–1 cm long tubes, bruising indigo blue at pore mouth and along tubes, 2 pores per mm.

Stipe 5–10 × 2–5 cm, yellow, lacking annulus, cylindrical to clavate, straight, sometimes curving to sinuous, solid, central, base subclavate to fusiform, conspicuous, testaceous, vinaceous, or maroon punctules present on stipe, most frequent at base, becoming large stains on stipe, then becoming longitudinally streaked towards apex of stipe, sometimes becoming finely scabriform or flocciform, especially midway to apex of stipe; upper 1–3 mm of the stipe occasionally reticulate, becoming pronounced at maturity; mycelia below stipe base white.

Context firm, whitish to pale yellow, immediately cyanescent, especially in stipe and near tubes, this cyanescence appearing marbled against context, masking pale yellow pigment in context, deep red pigment present at base of stipital context.

Macrochemical reactions. KOH yellow to maroon on pileus, maroon on pores and stipe; NH_4OH yellow to yellow-orange on pileus, stipe, and context, negative on pore mouths (inducing indigo stain, then fading); $FeSO_4$ yellow to olive on stipe, negative elsewhere, bleaching blue stain from hymenophore.

Taste mild to slightly citrusy acidic sour. Odour mild, sometimes faintly fruity or citrusy. Basidiospores olive-brown in fresh deposit.

Dried basidiome. Pileus smooth, glabrous, golden yellow-brown, with brownish red punctules, some becoming black, punctules never more than 0.5 mm diameter, pileus convex. Tubes free from stipe, not separable individually. Stipe golden yellow, sometimes upper portion of stipe

reticulate, punctules maroon to black, red colors most prominent at base, scabriform punctules blackish in upper portion.

Microscopic features. Basidiospores (12)13.8–15.9(18) × (4)4.6–5.8(7) µm (48 spores counted, Q = 2.85), straw-yellow in KOH and water, ellipsoidal to subellipsoidal, sometimes subfusiform, smooth, thin-walled, with a pronounced apiculus and rounded apex, having one, two, or three olive-colored oil droplets.

Basidia 20–30 × 10–16 µm, clavate to subclavate, thin-walled, hyaline, with yellow-green oil guttules in water and KOH, without basal clamps, predominantly four-spored, occasionally two-spored or three-spored; sterigmata 1–3 µm long; basidioles clavate to subclavate, size similar to basidia.

Cheilocystidia 20–32 × 6–8.5 µm, abundant, typically filiform to subclavate, ventricose, sometimes substrangulated, flexuous, cylindrical, apices subclavate to filiform, sometimes aciculate. Pleurocystidia shape and size similar to cheilocystidia, but more commonly ventricose to filiform.

Hymenophoral trama bilateral-divergent appearing subparallel in mature specimens, boletoid, lateral strata somewhat gelatinised, elements 7–15 µm wide, mediostrata gelatinised, loosely arranged, reddish brown, hyphae 7–15 µm wide.

Pileipellis an interwoven trichoderm, sometimes suprapellis collapsing into a cutis, elements filiform, sinuous, not constricting at septa, terminal elements (3)4–9(12) µm wide, some elements pigmented maroon-red, cylindrical, filiform, occasionally clavate, occasionally embedded or encrusted with yellow-green oil guttules, subterminal elements similar in size and shape to suprapellis.

Stipitipellis consisting of parallel to subparallel and longitudinally running, smooth-walled, septate hyphae, 4–6 µm wide, stipitipellis elements occasionally breaking up into pigmented (reddish brown in H_2O and KOH) fascicles arranged in anticlinal bundles, these elements terminating into subclavate to clavate elements, 5–10 µm diameter, 20–30 µm long.

Caulocystidia similar to pleurocystidia, but occasionally filiform, sinuous to flexuous, 50–100 × 5–6 µm; substipitipellis longitudinally interwoven; stipe stratum composed of 4–6 µm diameter septate hyphae, hyaline in H_2O and KOH, with occasional pigmented hyphae (reddish brown in H_2O and KOH) traversing stipe, and occasionally interwoven with stipe stratum, these hyphae 12–15 µm diameter.

Hyphal system monomitic. Clamp connections absent.

Ecology and distribution. Solitary to gregarious, beneath *Quercus* spp., predominantly in disturbed habitats during summer months. Known from peninsular Florida to Texas, common (Fig. 9).

2.4 Discussion

2.4.1 Phylogenetic position of the genus *Pulchroboletus*

Boletus rubricitrinus does not belong to the genus *Boletus*, according to our molecular analyses (Figs. 1–3). It appears that *B. rubricitrinus* is not a member of the subfamily *Boletoideae* (Nuhn et al. 2013, Wu et al. 2014) and is better placed in the genus *Pulchroboletus* Gelardi, Vizzini & Simonini. *Pulchroboletus* is in the *Hypoboletus* group in the subfamily *Xerocomoideae* of *Boletaceae* (Binder and Hibbett 2006, Šutara 2008, Nuhn et al. 2013; Wu et al. 2014).

Xerocomoideae contains boletoid and phylloporoid species; most often the pileipellis is a trichoderm. *Xerocomoideae* was erected as a subfamily by Singer (1945b), originally based on the *Phylloporus* Quél. hymenophoral trama. Pegler & Young (1981) raised this subfamily to the family

level (*Xerocomaceae*). Molecular evidence has brought this group back again to the subfamily level (Binder & Hibbett 2006, Nuhn et al. 2013, Wu et al. 2014).

Alessioporus Gelardi, Vizzini & Simonini and *Pulchroboletus* are two genera erected to accommodate two Mediterranean species formerly placed in *Xerocomus* Quél., *X. ichnusanus* Alessio, Galli & Littini and *X. roseoalbidus* Alessio & Littini, respectively (Gelardi et al. 2014). Recently, Frank et al. (2017) described a novel Eastern North American species in *Alessioporus,* based on ITS sequences. *Hemileccinum* Šutara is a related genus with five species currently known (Šutara 2008, Halling et al. 2015, Wu et al. 2016) and is similar to *Alessioporus* and *Pulchroboletus*, but differs in the presence of very fine scales on the stipe, violet reaction with ammonia on the pileus, and a presence of an iodine-like odour at the base.

2.4.2 Delimitation of *Pulchroboletus* species

Pulchroboletus is characterised by a rosy-colored pileus which is hemispherical and becoming flattened to uplifted at maturity, a yellow tubulose hymenophore which bruises blue, and a smooth to fibrillose yellow-orange stipe with basal maroon punctuations. Both species of *Pulchroboletus* can be found in warm climates, and while both are associated with *Quercus* spp., *Pulchroboletus roseoalbidus* also associates with *Castanea* and *Cistus*. *Pulchroboletus roseoalbidus* tends to grow in caespitose clusters, while *P. rubricitrinus* tends to grow gregariously.

Morphological similarities exist between *P. roseoalbidus* and *P. rubricitrinus*. Both have a pinkish red cuticular color on the pileus, but *P. roseoalbidus* exhibits a much paler pink pileus. The pileus diameter in both species overlap, with *P. rubricitrinus* occasionally expanding to 15 cm diameter; both are hemispherical to convex, becoming applanate to somewhat uplifted at maturity. Both pileus cuticles are subtomentose to glabrous, non-viscid, dry, and somewhat greasy with moisture. The tubes of both species are depressed, then become decurrent with a tooth. The

spore print of both species is olive-brown. Basidiospores of both species exhibit similar shapes, and are one-, two-, or three-guttulate. Singer (1986) reported the KOH reaction as deep red on the pileus and brown elsewhere in *P. rubricitrinus*; our observations indicate a reddish brown on the pileus and a deep (maroon) red on the pore surface. Application of KOH to *P. roseoalbidus* results in a pinkish color on the pileal context, orange on the stipe context, and reddish brown at the base of the stipe.

The main distinguishing morphological feature between these two species are the maroon floccules present on the stipe of *P. rubricitrinus*, which are present as mere punctules in *P. roseoalbidus*. Another distinguishing feature is the context color; the pileus context of *P. roseoalbidus* is lilac-pinkish while the pileal and stipe contexts in *P. rubricitrinus* are whitish yellow, and maroon red at the base of the stipe. The granular pseudoannular zone on the stipe of *P. roseoalbidus* is not present in *P. rubricitrinus*. Cystidia in *P. rubricitrinus* are generally shorter in length than *P. roseoalbidus*.

The reaction of NH_4OH differs between the two species. It is rusty brown on the hymenophore, orange on the stipe, and negative elsewhere (bleaching lilac-pink context color away) on *P. roseoalbidus*; NH_4OH on *P. rubricitrinus* reacts yellow to orange on the pileus, pores and context, and brown on the stipe. *Pulchroboletus roseoalbidus* exhibits olive colors with the application of $FeSO_4$ on all tissues; *P. rubricitrinus* exhibits a yellow color on the stipe, negative elsewhere, and bleaching blue color from stained hymenophore.

While *P. roseoalbidus* is found in the Mediterranean, data from mycoportal.org (Fig. 9, Appendix) shows that *P. rubricitrinus* is distributed from Florida to Texas. We have not verified these identifications from mycoportal.org, although a photograph from Texas in Metzler & Metzler (2010: 209) is consistent with the diagnostic macromorphological features of *P. rubricitrinus*.

Most specimens on mycoportal.org were found beneath *Quercus virginiana, Q. laurifolia*, or *Quercus* spp. One collection was beneath *Pinus* as well as *Quercus* spp. (H. Luke, s.n., 11 June 2000). *Pulchroboletus rubricitrinus* is likely mycorrhizal with *Quercus virginiana* and *Q. laurifolia*. Our observations (records included in Appendix) indicate that *P. rubricitrinus* is typically found in lawns beneath or near *Quercus* spp., and not in treeless lawns. Gelardi et al. (2014) considered both *Alessioporus* and *Pulchroboletus* to be mycorrhizal.

2.4.3 Potentially related species

Five specimens collected by Rolf Singer in Miami-Dade Co. and originally identified as *Boletus rubricitrinus* were excluded from our analyses as these likely represent collections of *B. fairchildianus* (Singer) Singer. *Boletus fairchildianus* was first described as *B. rubricitrinus* var. *fairchildianus* Singer (Singer 1945) and later elevated to the species level (Singer 1977). Although *B. fairchildianus* is similar to *P. rubricitrinus*, we cannot be certain if *B. fairchildianus* is closely related to it, especially without DNA sequences. Singer (1945) notes that it differs from *P. rubricitrinus* by its red pore mouths. However, photographs identified by Bessette et al. (2016: 104) as *B. fairchildianus* show a redder stipe which is less floccose and less reticulated, exhibits darker bruising, and a more variable color of red in the pileus.

This study has also identified three unknown environmental bolete sequences from GenBank which may belong in *Pulchroboletus* (Tab. 1, Fig. 1). The sequence EU569326.1 was from a specimen found in a cloud forest in tropical Mexico (Morris et al. 2008). The sequence FM999554.1 was from an uncultured environmental sample from a beech-maple forest in Ohio, USA (Burke et al. 2009). The sequence FJ480444.1 was from a bright orange sclerotium collected in Massachusetts, found near the sclerotium of a *Boletus rubropunctus* Peck specimen (Smith & Pfister 2009); Smith and Pfister postulated that despite being present in ancestral bolete lineages,

sclerotium growth was lost by many taxa in the *Boletales*, and has resurfaced as a convergent trait in the suborders *Boletineae* and *Suillineae*. This indicates the first-known sclerotium-forming species in the *Hypoboletus* group.

2.5 Conclusion

This paper updates our understanding of the taxonomy of *Pulchroboletus rubricitrinus* in the light of DNA phylogenetics and provides the first sequences of this bolete. A thorough morphological description is now also available, and an epitype has been established. These molecular and morphological data will be useful to further improve our understanding of taxonomic groups during this period of rapid bolete reclassification.

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Table 2.1. Sequences used for phylogenetic analyses. Taxon names correspond to those listed in GenBank.

Taxon	**Origin**	**ITS GenBank No.**	**LSU GenBank No.**	**Voucher No.**	**References**
Alessioporus ichnusanus	Corsica, France	KJ729498	KJ729511	TO AVX13	Gelardi et al. 2014
Alessioporus ichnusanus	Lazio, Italy	KJ729496	KJ729509	MG 420a	Gelardi et al. 2014
Alessioporus ichnusanus	Piedmont, Italy	KJ729495	KJ729508	RG XER.ICH 6	Gelardi et al. 2014
Alessioporus ichnusanus	Lazio, Italy	KJ729493	KJ729506	MG 549a	Gelardi et al. 2014
Alessioporus rubriflavus	Suffolk Co., New York, USA	KU736957	—	ARB1356	Frank et al. 2017
Alessioporus rubriflavus	Suffolk Co., New York, USA	KC812305	KC812206	JLF2561	Frank et al. 2017
Alessioporus rubriflavus	Oconee Co., South Carolina, USA	KU736958	—	JLF2561b	Frank et al. 2017

Alessioporus rubriflavus	Elbert Co., Georgia, USA	KT223008	KT223009	ARB1262	Frank et al. 2017
Boletus rubricitrinus	Sarasota Co., Florida, USA	MF193883		USF Franck 3114	This study
Boletus rubricitrinus	Hillsborough Co., Florida, USA	MF193884	MG026638	USF Farid 335	This study
Boletus rubricitrinus	Hillsborough Co., Florida, USA	MF193885	—	USF Franck 3473	This study
Boletus rubricitrinus	Taylor Co., Florida, USA	MF193886	—	USF Franck 3594	This study
Boletus sp.	Guerrero, Mexico	EU569236	—	UC MHM075	Morris et al. 2008
Boletus sp.	Middlesex Co., Massachusetts, USA	FJ480444	—	FH MES260	Smith & Pfister 2009
Pulchroboletus roseoalbidus	Sardinia, Italy	KJ729486	KJ729499	AMB 12757	Gelardi et al. 2014
Pulchroboletus roseoalbidus	Lazio, Italy	KJ729487	KJ729500	MG 532a	Gelardi et al. 2014
Pulchroboletus roseoalbidus	Lazio, Italy	KJ729489	KJ729502	MG 416a	Gelardi et al. 2014
Pulchroboletus roseoalbidus	Emilia Romagna, Italy	KJ729490	KJ729503	MCVE 17577	Gelardi et al. 2014
Xerocomus depilatus	—	AY127032	AF139712	—	Unpublished
Xerocomus impolitus	Portugal	HM347651	—	UF1464	Unpublished
Xerocomus impolitus	Spain	HM347650	AF139715	JAM0585	Unpublished
Uncultured fungus	Ohio, USA	FM999554	—	isolate S0681	Unpublished

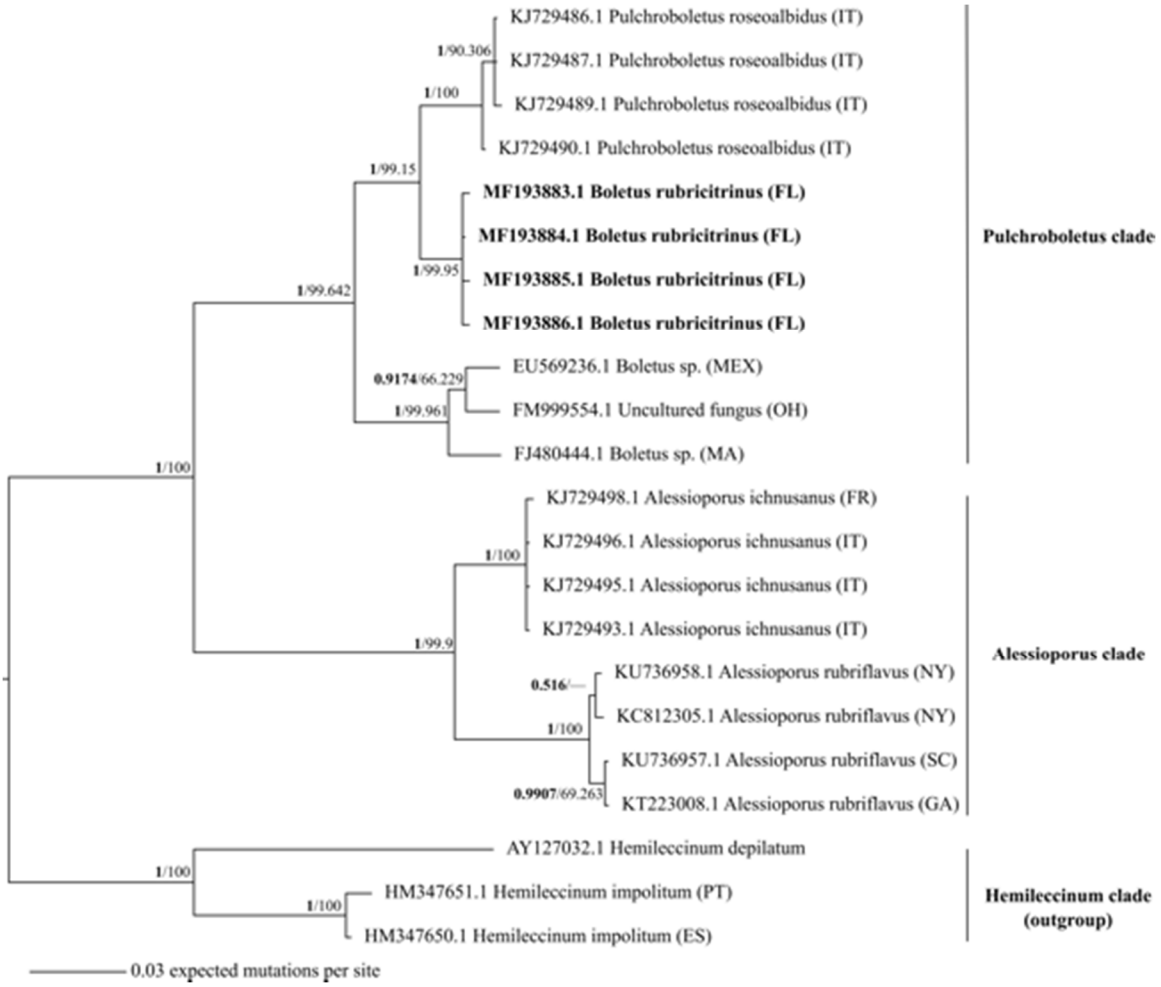

Fig. 2.1. Bayesian tree inferred from ITS sequences. BPP values exceeding 0.5 and ML bootstrap values exceeding 50% are shown adjacent to nodes. GenBank numbers precede the taxon names provided by GenBank, followed by the location of the collection. Novel sequences from this study are in bold. Abbreviations: IT = Italy, FL = Florida, MEX = Mexico, OH = Ohio, MA = Massachusetts, FR = France, NY = New York, SC = South Carolina, GA = Georgia, PT = Portugal, and ES = Spain; no locality data could be obtained for AY127032, although it is likely from Europe.

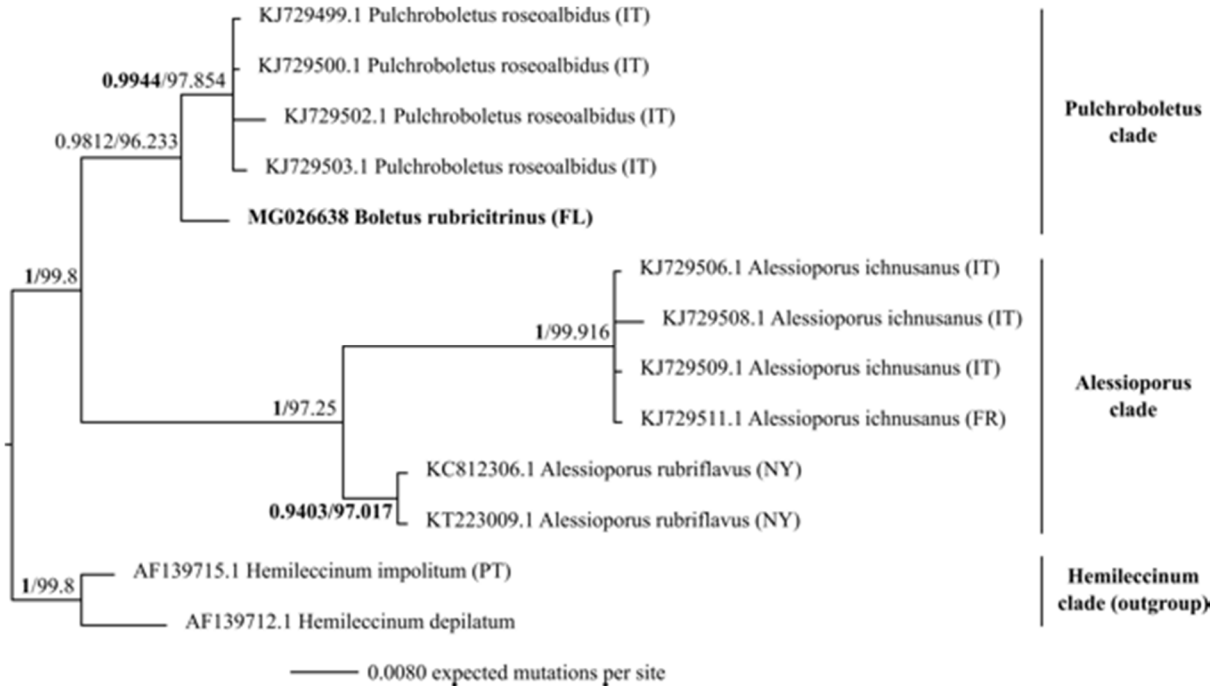

Fig. 2.2. Bayesian tree inferred from LSU sequences. BPP values exceeding 0.5 and ML bootstrap values exceeding 50% are shown adjacent to nodes. LSU GenBank numbers precede the taxon names provided by GenBank, followed by the location of the collection. The novel LSU sequence from this study is in bold. Abbreviations: IT = Italy, FL = Florida, FR = France, NY = New York, and PT = Portugal; no locality data could be obtained for AF139712, although it is likely from Europe.

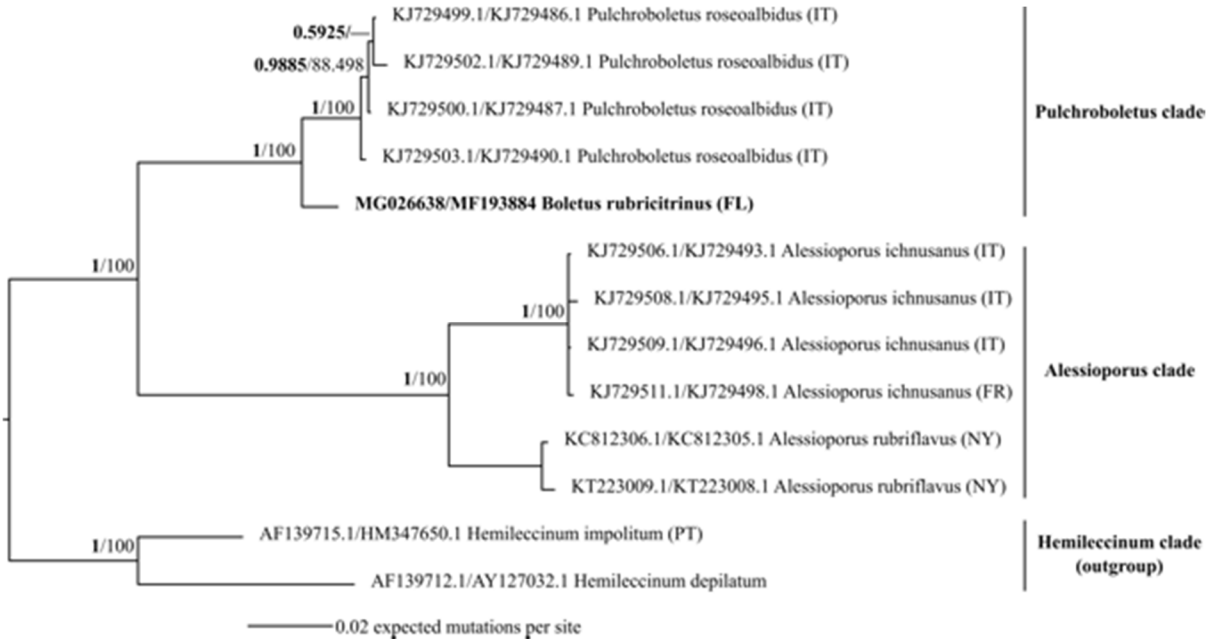

Fig. 2.3. Bayesian tree inferred from combined LSU and ITS sequences. BPP values exceeding 0.5 and ML bootstrap values exceeding 50% are shown adjacent to nodes. LSU/ITS GenBank numbers precede the taxon names provided by GenBank, followed by the location of the collection. The novel LSU/ITS sequence from this study is in bold. Abbreviations: IT = Italy, FL = Florida, FR = France, NY = New York, and PT = Portugal; no locality data could be obtained for AF139712/AY127032, although it is likely from Europe.

Fig. 2.4. Field photograph of *Pulchroboletus rubricitrinus* (Franck 3473). Photograph by A.R. Franck.

Fig. 2.5. Field photograph of *Pulchroboletus rubricitrinus* (Farid 335). Photograph by A. Farid.

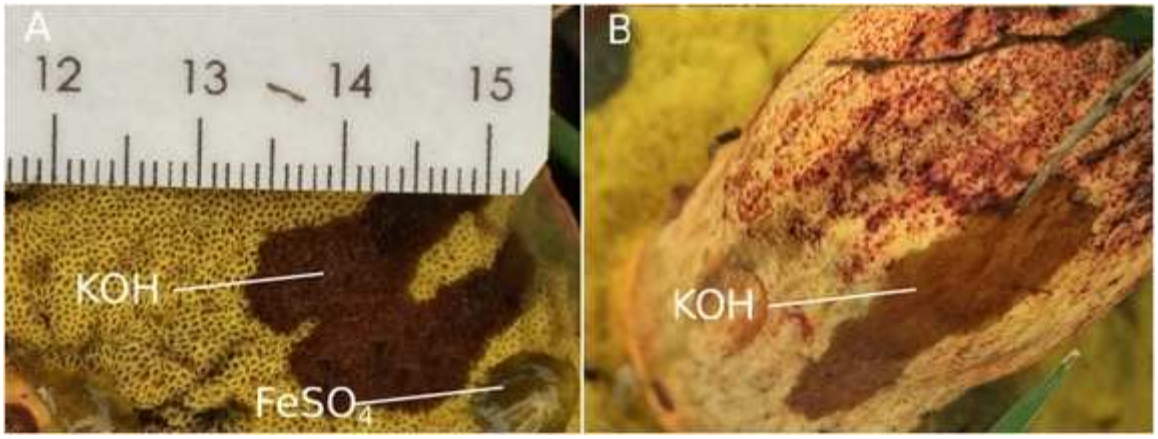

Fig. 2.6. Field photographs of *Pulchroboletus rubricitrinus* (Farid 335). **A** – hymenophore; **B** – flocciform punctuations at stipe base. Photographs by A. Farid.

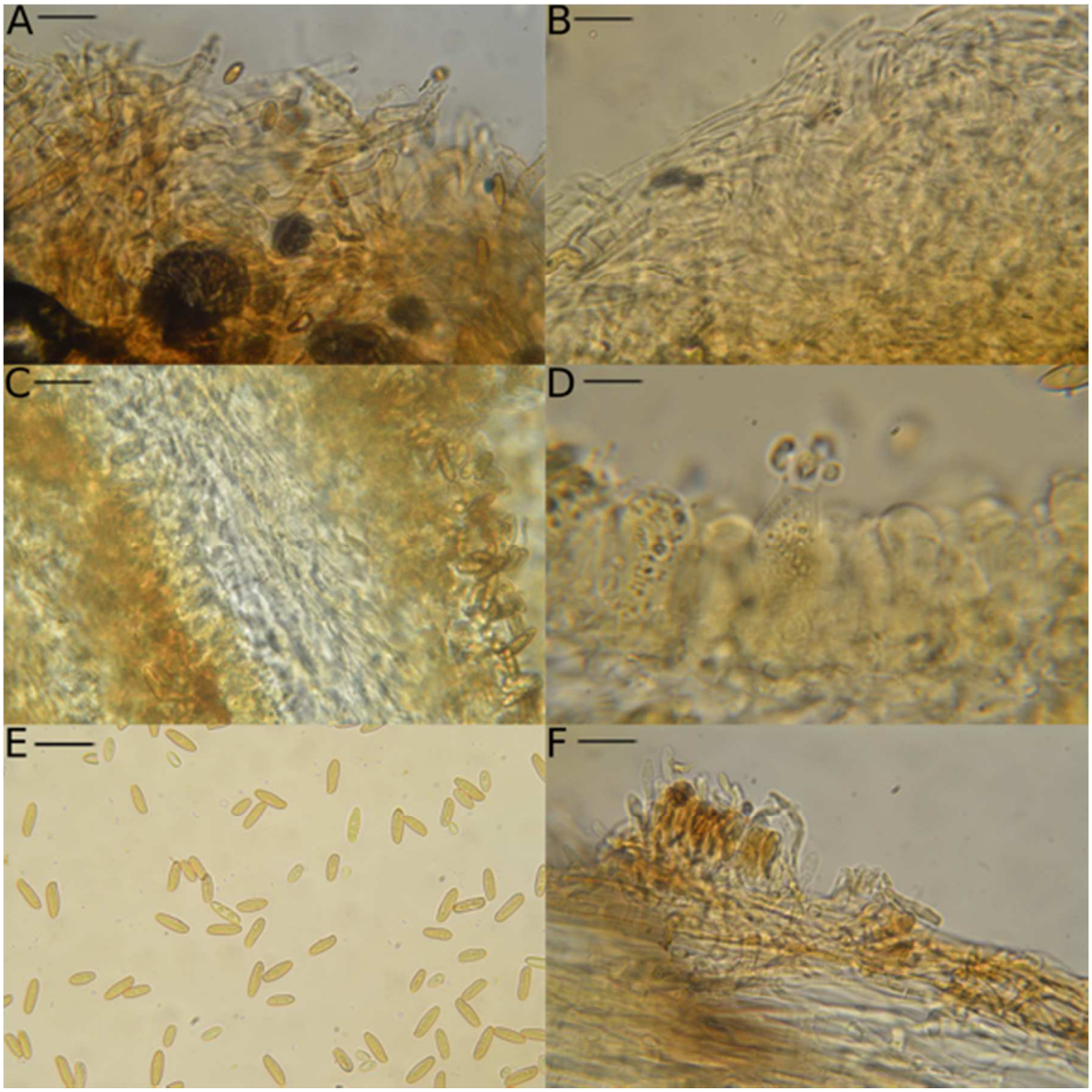

Fig. 2.7. Microscopic features of *Pulchroboletus rubricitrinus*. **A** – pileipellis a trichoderm (Franck 3594); **B** – pileipellis a cutis (Farid 335); **C** – hymenophoral trama (Franck 3473); **D** – basidia and basidioles (Franck 3473); **E** – basidiospores (Farid 335); **F** – fascicles arising from stipitipellis (Farid 335). Scale bars = 15 µm (A–C, E–F), 30 µm (D). Photographs by A. Farid.

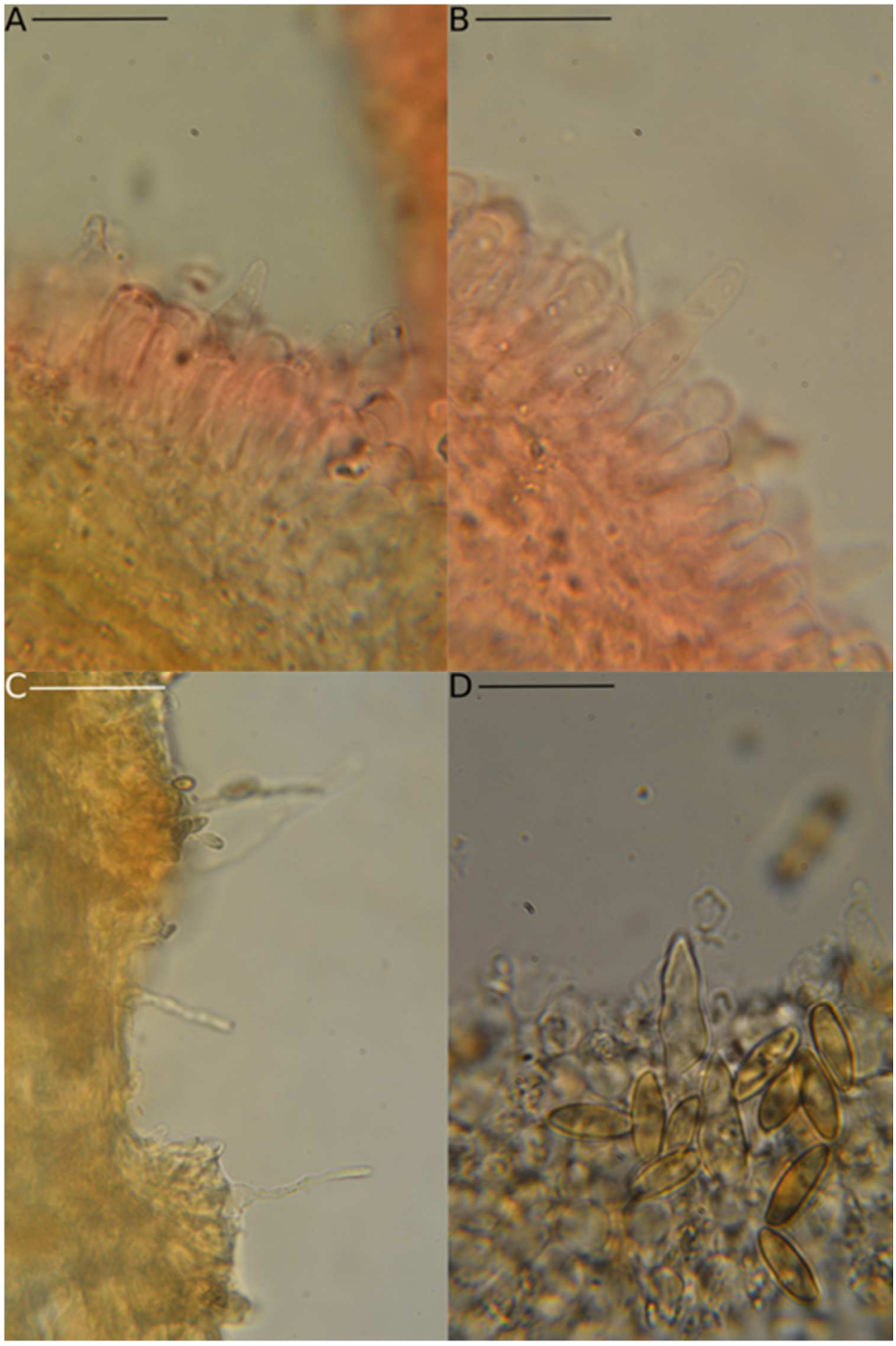

Fig. 2.8. Microscopic features of *Pulchroboletus rubricitrinus*. **A, B** – pleurocystidia; **C** – caulocystidia (Franck 3036); **D** – pleurocystidia (Franck 3473). Scale bars = 30 µm. Photographs by A. Farid.

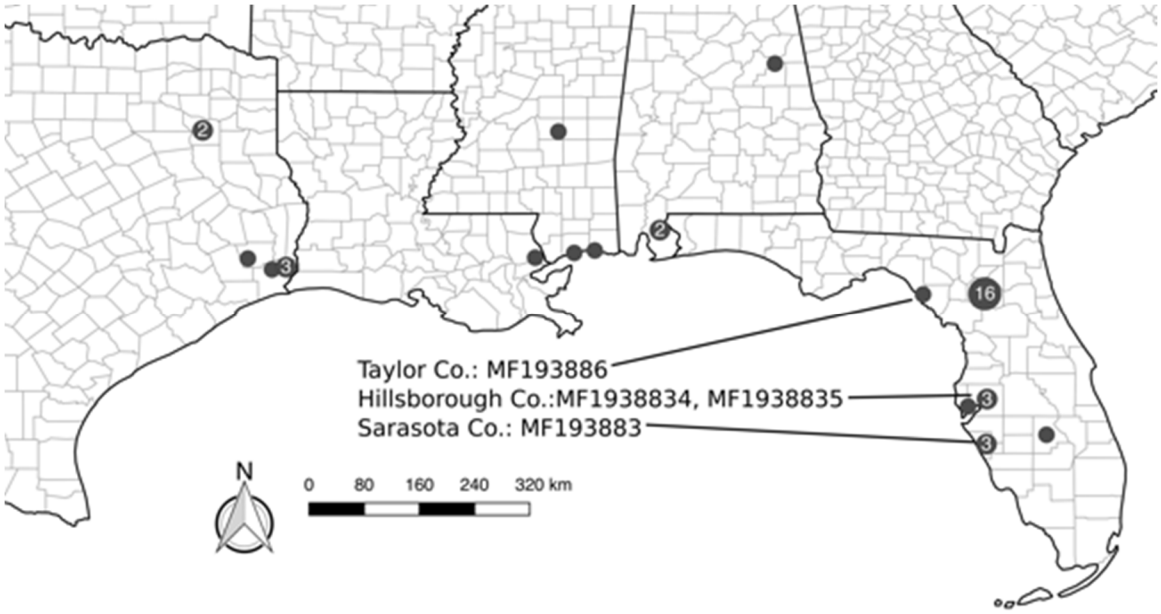

Fig. 2.9. Map generated from Mycoportal.org data download using QGIS (version 2.18.2). Counties with more than one collection are shown with numbers indicating the number of collections reported, and visualised as county centroids. Coordinate reference system: EPSG:54032.

CHAPTER THREE: THE NOVEL GENUS *PSEUDOPHYLLOPORUS* (BOLETACEAE) TO ACCOMMODATE *PHYLLOPORUS BOLETINOIDES*

Note to reader. Portions of this work are currently in review for the journal Mycosphere.

3.1 Introduction

Phylloporus Quél. is a genus of lamellate fungi in the family Boletaceae (Kirk et al. 2008). Quélet (1888: 409) placed *Phylloporus* with the boletes (Trib. III Boleti), in the polypore family (Fam. V Polyporei). Quélet's series Paradoxi (Sér. I), contained *Phylloporus*, along with anastomosing genera of boletes *Euryporus* Quél. (=*Suillus*) and *Uloporus* Quél. (=*Gyrodon*). Lotsy (1907:716) placed *Phylloporus* and the genus *Paxillus* Fr. in the family Paxillaceae because of: (1) its microscopic features, (2) its lamellae, which are readily detached from the pileus, and (3) its bolete-like growth habit. Singer (1945) moved *Phylloporus* to the subfamily Xerocomoideae Singer (Boletaceae) due to its *Phylloporus*-type hymenophoral trama and olive-brown spore print. Bresinsky & Besl (2003) synonymized *Phylloporus* with *Xerocomus* Quél., although only a few taxa were sampled in that study. Recent molecular analyses with more extensive sampling support the monophyly of *Phylloporus*, show *Xerocomus* is highly polyphyletic (Binder & Bresinsky 2006, Šutara 2008, Wu et al. 2014, Wu et al. 2016), and support the placement of *Phylloporus* in an expanded Xerocomoideae that now also includes taxa with *Boletus*-type hymenophoral trama and pale yellow to brown spore prints (Šutara 2008, Wu et al. 2014, Wu et al. 2016).

Neves and Halling (2010) estimated the genus *Phylloporus* contains about 70 species, with new species being described from under-sampled regions (Neves et al. 2010, Zeng et al. 2013,

Hosen & Li 2017). *Phylloporus* is primarily distributed throughout the tropics (Kirk et al. 2008, Neves & Halling 2010). Species in *Phylloporus* form ectomycorrhizal associations with plants in the families Casuarinaceae, Dipterocarpaceae, Fabaceae, Fagaceae, Myrtaceae, and Pinaceae (Neves & Halling 2010, Neves et al. 2012, Zeng et al. 2013, Hosen & Li 2017). North America is known to contain five species of *Phylloporus* (Neves 2007, Neves et al. 2010). Though most species of *Phylloporus* exhibit the *Phylloporus*-type hymenophoral trama, there are several species which exhibit the *Boletus*-type hymenophoral trama, such as the Australasian species *P. australiensis* and *P. cingulatus* (Watling & Gregory 1991) and neotropical species *P. boletinoides* Smith & Thiers and *P. fibulatus* Singer, Ovrebo & Halling.

Phylloporus boletinoides is a lamellate bolete, and can be clearly differentiated from other *Phylloporus* species by its olive-toned lamellae that are relatively more anastomose (Smith & Thiers 1964) and its *Boletus*-type hymenophoral trama. *Phylloporus boletinoides* was described from a collection in Alachua Co., Florida, USA, beneath *Pinus*. Singer et al. (1990) redescribed the type collection and placed the species in *Phylloporus* sect. *Fibulati* Singer, Overbo, & Halling.

Though the morphology of *P. boletinoides* has been studied thoroughly and suggests placement in *Phylloporus* (Smith & Thiers 1964, Singer et al. 1990, Neves 2007, Neves & Halling 2010), this species has not been analyzed using molecular tools. In this study, we examine the taxonomic placement of this species in a molecular context.

3.2 Materials & Methods

3.2.1 Collection sites and sampling

Specimens examined were collected in peninsular Florida and deposited at the University of South Florida Herbarium (USF) and the Florida Museum of Natural History at the University of Florida (FLAS). One specimen was obtained on loan from USDA Forest Service, Center for Forest

Mycology Research (CFMR). Specimens were identified based on Smith & Thiers (1964) and keys from the literature (Neves & Halling 2010, Bessette et al. 2016).

3.2.2 Morphology

Macroscopic features are based on detailed notes from fresh basidiomes. Micromorphological features were observed from dried specimens using a compound microscope (AccuScope, Commack, NY, USA). Distilled H_2O, 5% KOH, phloxine, and Melzer's reagent were used to rehydrate and stain sections. Measurements were made at 1000× with a calibrated ocular micrometer using Piximètre 5.9 (http://www.piximetre.fr). Micrographs were taken using a NIKON D3200 camera. Basidiospore dimensions were reported as length by width; each measurement was reported as the minimum, the average minus the standard deviation, the average plus the standard deviation, and the maximum. Measurements are followed by the average quotient Q, where Q = average length divided by average width, and N, the number of spores counted.

3.2.3 DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was extracted from dried herbarium specimens using a modified CTAB extraction protocol (Doyle & Doyle 1987, Franck et al. 2012) and diluted in 65 µl of a 10 mM Tris, 1 mM EDTA buffer.

Amplification reactions were conducted on a T3 Thermocycler (Biometra, Göttingen, Germany) with 20 µl volumes using 1 unit ID*Proof*™ Taq Polymerase (Empire Genomics, Buffalo, NY, USA), 2 µl 10× Reaction Buffer, 3 mM $MgCl_2$, 120 ng of each primer, 250 µM dNTPs, and 1 µl of DNA. Serial dilutions were attempted if amplification failed. Amplicons were visualized in 0.9% agarose using TAE buffer and 1% ethidium bromide to ensure products of expected size were produced. Crude PCR product was purified and sequenced at the DNA Laboratory at Arizona State University using a 3730 DNA Analyzer (Applied Biosystems,

Carlsbad, CA, USA) using the same PCR primers. Contigs were generated based on sequence reads of the same loci.

The fungal-specific primers ITS1-F/ITS4 (Gardes & Bruns 1993) were used to amplify ITS1, 5.8S rRNA, and ITS2 (ITS). Amplification cycle parameters for the ITS region were as follows: 94 ºC for 3 minutes for initial denaturation, followed by 40 cycles of 94 ºC for 45 s, 51 ºC at 45 s for annealing, and an extension at 72 ºC for 90 s, with a final extension of 72 ºC for 5 minutes.

The primer pair LR0R/LR7 (Vilgalys & Hester 1990) was used to amplify 28S rRNA (LSU). Amplification cycle parameters for the LSU region were as follows: 95 ºC for 2 minutes for initial denaturation, followed by 40 cycles of 94 ºC for 45 s, 50 ºC for 70 s for annealing, and an extension at 72 ºC for 120 s, with a final extension of 72 ºC for 10 minutes.

Amplifications of the DNA-directed RNA polymerase II subunit 1 region (RPB1) were attempted using the fungal primer pair RPB1-Af/RPB1-Cr (Mathney et al. 2002) and bolete-specific primer pair RPB1-B-F1/RPB1-B-R (Wu et al. 2014), but were unsuccessful. To increase amplification success, RPB1 primers specific to the *Bothia/Solioccasus* clade inferred from Wu et al. (2014) were developed (Table 3.1). A touchdown PCR was used to amplify the RPB1 region with the primer pairs RPB1-32-F/RPB1-835-R and RPB1-147-F/RPB1-1091-R. The cycle parameters as follows for RPB1-32-F/RPB1-835-R: (1) 10 cycles of 94 ºC for 2 minutes, (2) 94 ºC for 40s, (3) 66 ºC for 40 s, minus 1 ºC every cycle, (4) 72 ºC 1 minute and 30 s, (5) repeat steps 2–4 for nine cycles, (6) 94 ºC for 45 s, (7) 56 ºC for 90 s, (8) 72 ºC for 90 seconds, (9) repeat steps 6–8 for 35 cycles, (10) 72 ºC for 300 s. For the primer pair RPB1-147-F/RPB1-1091-R, the annealing temperatures for steps (3) and (7) are 63 ºC and 53 ºC, respectively.

3.2.4 Phylogenetic analysis

Sequences obtained were run through the BLASTn algorithm (Boratyn et al. 2013) to identify closely related sequences. These sequences were combined from sequences in the literature (Binder & Hibbett 2006, Wu et al. 2014, Wu et al. 2016, Farid et al. 2017, Table 3.2) to create an alignment of the major groups in the Boletaceae for subsequent phylogenetic analysis. Global alignments for each gene were created using the MUSCLE algorithm (2004a, 2004b) in MEGA7 (Kumar et al. 2016) using default parameters. Sequence data was concatenated using Sequence Matrix (http://gaurav.github.io/taxondna/). Phylogenetic hypotheses were constructed using Bayesian inference (BI) and Maximum likelihood (ML) methods. Phylogenetic analyses were conducted separately for each gene (not shown), then as a concatenated dataset. Topology of individual phylogenies were congruent with each gene. Subsequently, a tree with a reduced dataset was produced to minimize potential effects of missing data. Alignments were run through Gblocks 0.91b (Castresana 2000, Calavera & Castresana 2007) to remove sites in the alignment which were not informative for phylogenetic analysis. The best-fit substitution models for both Bayesian information criterion (BIC) and Akaike information criterion (AICc) were conducted using jModelTest 2.1.10 (Guindon & Gascuel 2003, Darriba et al. 2012). The models provided were as follows: (1) for ITS, both BIC and AICc were HKY+I+G, (2) for LSU, both BIC and AICc were GTR+I+G, and (3) for RPB1, for BIC and AICc were HKY+I+G and K80+I+G respectively. The models provided for the reduced dataset by were as follows: (1) for ITS, BIC and AICc provided HKY+G and K80+G respectively, (2) for LSU, BIC and AICc provided GTR+I+G and K80+I respectively, and (3) for RPB1, BIC and AICc provided GTR+I and K80+I respectively. All subsequent phylogenetic analyses were conducted using the BIC models provided; analyses were also run using the AICc models, with no significant topological difference (not shown). BI was conducted with MrBayes version 3.2.6 (Ronquist et al. 2012) using four Markov chain Monte

Carlo 10,000,000 generations, sampling trees every 1,000 generations. The first 25% were discarded as burn-in, and a majority rule consensus tree was computed to obtain estimates for Bayesian posterior probabilities (BPP). ML was conducted with RAxML 8.2.11 (Stamatakis 2014) using 1,000 non-parametric bootstrap replicates. ML trees were visualized in Dendroscope version 3.4.9 (Huson 2012) with BS values displayed as node labels. For BI and ML trees, taxa missing target loci were encoded as missing data (Felsenstein, 2004). ML trees were then exported into Inkscape version 0.91 (http://www.inkscape.org) to add BPP values to node labels. BPP equal to and above 0.95 were reported, and BS values above 70% were reported. Alignment and phylogenetic trees were uploaded to http://treebase.org/ (submission ID 22222).

3.3 Results

3.3.1 Phylogenetic analyses

Three ITS sequences, one LSU sequence and one RPB1 sequence were obtained from three collections of *Phylloporus boletinoides* (Table 3.2).

The alignment for the family-wide tree consisted of 41 ITS sequences, 64 LSU sequences, and 38 RPB1 sequences comprising 70 OTUs. *Phylloporus boletinoides* formed a sister clade with 95% BS support and 0.99 BPP to the *Bothia* Halling, T.J. Baroni & M. Binder group (Fig. 3.1). *Bothia* and *Phylloporus boletinoides* formed a clade adjacent to *Solioccasus* Trappe, Osmundson, Binder, Castellano & Halling with 1 BPP and 95% BS.

The dataset of the reduced phylogenetic tree (Fig. 3.2) consisted of 13 ITS sequences, 12 LSU sequences, and 4 RPB1 sequences, corresponding to 16 OTUs. No topological differences were found between the ML and BI tree. In Fig. 3.2, the *Phylloporus boletinoides* clade is supported with 1.00 BPP and 100% BS support. The *Bothia* clade is weakly supported as a sister group to *Pseudophylloporus*, with 0.59 BPP and below 50% BS support.

Two environmental sequences were grouped in the *Phylloporus boletinoides* clade (Figs 3.1 & 3.2): KX899732, an environmental sequence from Jonathan Dickinson State Park, Hobe Sound, FL, USA, and KX899785, an environmental sequence from Big Lagoon State Park, Pensacola, FL, USA. Both sequences were obtained from ectomycorrhizal samples on *Pinus clausa* (Chapm. ex Engelm.) Sarg. (Sand Pine). 3.3.2 Taxonomy

Pseudophylloporus A. Farid & M.E. Smith, gen. nov. Figs 3–4

MycoBank number: MB 824049; Facesoffungi number: FoFXXX

Etymology – named for its similar appearance to the genus *Phylloporus*.

Basidiomes pileo-stipitate with lamellate to subporoid hymenophore, epigeal. *Pileus* appressed tomentose, brownish-red to light brown, with an even margin. *Hymenium* lamellate, interveinose to sub-anastomose, dingy olive-yellow, decurrent. *Stipe* cylindrical, golden brown, sometimes sinuous. *Context* white in pileus, staining indigo near the pileus and hymenophore, stipital context dingy yellow, more or less concolorous with the stipe. *Spore print* olive brown. *Basidiospores* smooth, subfusoid. *Cystidia* fusoid, ventricose. *Hymenophoral trama* of the *Boletus*-type. *Pileipellis* an interwoven trichoderm. *Stipitpellis* of hyaline longitudinal hyphae.

Type species – Pseudophylloporus boletinoides (A.H. Sm. & Thiers) A. Farid & M.E. Smith.

Notes – The coloration of the pileus, context, and stipe of this genus closely resemble *Bothia* T.J. Halling, Baroni & Binder. The dimensions of spore size, basidia, and hymenial cystidia overlap considerably in both genera, but the cystidia in *Bothia fujianensis* N.K. Zeng, M.S. Su, Z.Q. Liang & Zhu L. Yang are relatively longer. The pileipellei is a trichoderm of similarly sized hyphal elements in both *Pseudophylloporus* and *Bothia*. The hymenophoral trama of both genera are of the *Boletus*-type. *Pseudophylloporus* can be readily distinguished from *Bothia* by its drab olive-yellow lamellate hymenophore, which is tubulose and dark brown to dark rusty brown in

Bothia. The spore print in *Pseudophylloporus* is olive brown, while in *Bothia* it is a lighter yellow brown.

Pseudophylloporus boletinoides (A.H. Sm. & Thiers) A. Farid & M. E. Smith, comb. nov. Figs 3–4

Basionym: *Phylloporus boletinoides* A.H. Sm. & Thiers, Monogr. North Amer. Species Suillus: 105 (1964).

MycoBank number: MB 824072; Facesoffungi number: FoFXXXX

The following description is based on the following material: Baroni BZ-745, Farid 617, Franck 3125, Kraisitudomsook NAT-033, and Smith s.n. (see Appendix). These features are consistent with the type material (Smith & Thiers 1964, Singer et al. 1990, Neves 2007, Neves & Halling 2010).

Occurring singly in xeric hammock, sandy soil, near *Pinus* sp., *Quercus laurifolia*, and *Q. nigra*.

Pileus 4.5 cm diameter, convex, finely appressed floccose or tomentose, chestnut to brownish red, tan beneath, bruising blackish-blue, margins even, entire.

Hymenium lamellate, decurrent, distant to subdistant, interveinous, lamellulae-like structures present, olive yellow, bruising greenish blue, edges even, face 5 mm wide.

Context white, edges near cuticle and hymenium staining indigo blue to a purplish blue, context yellow at base of the stipe, hollowed in stipital context, this region discoloring brown.

Stipe 3 cm long x 7 mm wide, cylindrical, slightly curved, finely reticulate on the upper 3 mm, brownish red, golden striate, subclavate at base (becoming 8mm wide).

KOH dark reddish on all surfaces; NH4OH blood-red orange on pileus, negative lamellae, pink red context, stipe orange to blood-red; FeSO4 olive-gray on pileus and stipe, negative elsewhere.

Pileipellis a tightly interwoven trichoderm of cylindrical elements, these hyaline to pale yellow, yellowish brown in KOH and Melzer's, terminal branches 30–42 µm long × 5–7.5 µm wide, thin-walled to occasionally thick-walled (up to 1 µm thick), subfusoid, subclavate, cylindrical to slightly sinuous, elements occasionally filled with greenish oil droplets, elements occasionally branching.

Context tightly interwoven, hyaline, elements 5–10 µm wide, septate, sometimes constricted or inflated at septa; nests of 5–25 µm plasmatic cells occasionally present, these concolorous with pileipellis pigments, turning orange-red in phloxine (with NH4OH).

Hymenophoral elements 5–8 µm wide, inamyloid, hyaline, of the *Boletus*-type arising from a somewhat gelatinized mediostrata, subhymenium layer a narrow band of small, cellular elements, 2–4 µm wide.

Basidia 30 µm long × 9 µm wide, 4-spored, subclavate to cylindrical, inamyloid, pinkish in phloxine (with NH4OH), sometimes filled with small, greenish oil droplets, sterigmata 1–3 µm long. Basidioles similar in size and appearance, generally 1–5 µm shorter.

Basidiospores (8.1) 8.8–10.2 (10.7) × (3.3) 3.6–4.5 (5.7) µm Q = (1.7) 2–2.7 (3); N = 30, pale brown in KOH, tawny in Melzer's, smooth, thin-walled 0.5 µm to occasionally 0.8 µm thick, face view subcylindrical to narrowly oval, in profile view inequilateral, somewhat subfusoid.

Pleurocystidia 60–70 µm long × 12–14 µm wide, hyaline, thin-walled, ventricose, apices obtuse, subclavate, rarely somewhat mucronate and thick-walled (to 1 um). Cheilocystidia similar in size and shape.

3.4 Discussion

Our phylogenetic analyses (Figs 3.1 & 3.2) support the creation of the novel monotypic genus *Pseudophylloporus* to accompany the species *Phylloporus boletinoides. Pseudophylloporus* is characterized by its drab olive-yellow, lamellate, interveinose hymenium.

Pseudophylloporus boletinoides is closely allied with the genera *Bothia* and *Solioccasus* (Figs 1 & 2). The genus *Bothia* was created to accommodate the species *Boletinus castanellus* Peck based on the highly divergent ITS and LSU sequences (Halling et al. 2007). The unique combination of morphological features caused much taxonomic confusion regarding this species, resulting in its placement in six different genera before its current placement in *Bothia*. *Bothia* was expanded to include the novel species *B. fujianensis* N.K. Zeng & Zhu L. Yang (Zeng et al. 2015). Though *Pseudophylloporus* and *Bothia* share many macromorphogical characteristics, the hymenium of *Bothia* is tubulose to boletinoid, but never lamellate as in *Pseudophylloporus*. The genus *Solioccasus* was erected for the novel species *S. polychromus* Trappe, Osmundson, Binder, Castellano & Halling (Trappe et al. 2013), a colorful sequestrate fungus. *Pseudophylloporus* differs from *Solioccasus* by its lamellate hymenium (sequestrate in *Solioccasus*) and its drab colors (brightly colored in *Solioccasus*).

All species in this clade, except for *B. castanella,* represent primarily tropical species of fungi. *Bothia fujianensis* occurs in southeastern China, while *Bothia castanella* is found in Eastern North America in the temperate zone. *Solioccasus* is known only from tropical and subtropical Australasia. Data from mycoportal.org show the distribution of *Pseudophylloporus boletinoides* (Fig. 5) is primarily tropical to subtropical, extending from Florida through to Texas. Three vouchers were collected from Belize. One sample (Baroni BZ-745, Appendix) was obtained on loan, and matched morphologically, though repeated PCR attempts produced no amplicon. This species is also present in temperate latitudes along the eastern USA; there are four observations

from mushroomobserver.org (MO 54732, 73726, 74400, and 10687), and one vouchered specimen (Halling 3811) from New Jersey (MO 106346). One mushroomobserver.org observation is from Delaware. All macromorphological features in photographs are consistent with those of *Pseudophylloporus boletinoides.*

Data obtained from top BLASTn results on GenBank (Figs 3.1 & 3.2) provide molecular confirmation of the ectomycorrhizal association of *P. boletinoides* with *Pinus*. GenBank sequences KX899732 and KX899785 were obtained from ectomycorrhizal samples on *Pinus clausa*. *Pseudophylloporus boletinoides* differs ecologically from *Bothia* and *Solioccasus* in terms of host preference. *Solioccasus polychromus* is associated with Myrtaceae (Trappe et al. 2013), and *Bothia fujianensis* grows in association with Fagaceae. *Bothia castanella* also grows in association with Fagaceae, but also occurs with *Betula* L.*, Pinus strobus* L., and *Tsuga canadensis* (L.) Carrière nearby.

The creation of the genus *Pseudophylloporus* suggests that a lamellate hymenium has evolved at least three times in the Boletaceae from poroid ancestors, occurring in the genera *Pseudophylloporus, Phylloporus*, and *Phylloboletellus* Singer. *Phylloporus* is in the subfamily Xerocomoideae, and is closely allied with *Xerocomus*; both genera are defined by their *Phylloporus*-type hymenophoral trama, but *Phylloporus* differs primarily by its lamellate hymenium. *Pseudophylloporus* is lamellate like *Phylloporus* but differs by its drab colors and its *Boletus*-type lamellular trama. *Phylloboletellus*, which contains the single species *Phylloboletellus chloephorus* Singer is known only from six collections; four from Argentina, and two from Mexico. Singer published the collections from Mexico as *Phylloboletellus chloephorus* var. *mexicana* ad int. Singer, J. García & L.D. Gómez, nom. inval., see Art 36.1 (Singer et al. 1992). Singer suspected that *Phylloboletellus* was closely allied with *Boletellus* Murrill (1986:785,

1992:45). *Phylloboletellus chloephorus* can readily be distinguished from other lamellate boletes by its longitudinally striate spores, olivaceous spore print, and *Boletus*-type lamellular trama. One LSU sequence of *Phylloboletellus chloephorus* (GB: DQ534658) exists from a strain which was isolated from a coffee plantation in Veracruz, Mexico. Phylogenetic analyses from our study (Fig. 3.1) and a previous study by Binder and Hibbett (2006) suggests that though this species is in the Boletaceae, it cannot yet be reliably placed in any named subfamily, nor is it closely allied to *Boletellus*.

3.5 Conclusion

This paper updates the taxonomic placement of *Psuedophylloporus boletinoides* using molecular analyses, and provides sequences of the ITS, LSU, and RPB1 regions; the first named sequences of this taxon on GenBank. This paper also highlights lamellae evolution in the Boletaceae. These molecular and morphological data will be useful for furthering our understanding of the interesting evolution of the boletes.

3.6 References

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Table 3.1 Primer design for *Bothia/Solioccasus* clade.

Primer name	Sequence (5' → 3')
RPB1-32-F	AGGCYGATATCGTGAGTCGC
RPB1-147-F	CTCGAGYTATCGAGGCGT
RPB1-835-R	ACCCTCRTCYTCRTCCTTGGG
RPB1-1091-R	CCATCYACYGCTATACTCGG

Table 3.2 DNA sequences used for the phylogenetic analyses (those generated in this study are in bold).

		GenBank Numbers		
Taxon Name	**Voucher ID**	**ITS**	**LSU**	**RPB1**
Austroboletus gracilis	112 96	—	DQ534624	KF030358
Baorangia bicolor	MB07-001	—	KF030246	KF030370
Baorangia pseudocalopus	HKAS 63607	—	KY418895	KJ184564
Baorangia pseudocalopus	HKAS75739	—	KY418895	KJ184564
Boletellus ananas	TH8819	—	HQ161853	HQ161822
Boletellus indistinctus	HKAS80681	—	KT990532	KT990903
Boletellus indistinctus	HKAS90215	—	KT990533	KT990904
Boletellus piakaii	TH8077	KT339225	HQ161861	HQ161830
Boletus aereus	REH8721	—	KF030339	KF030377
Boletus edulis	BD380	EU231984	HQ161848	KF030362
Boletus pallidus	179 97	DQ534564	AF457409	—
Boletus reticuloceps	HKAS62910	JN563884	JN563843	JN563862
Boletus rufomaculatus	4414	—	KF030248	KF030369
Boletus sinoedulis	HKAS55436	—	JN563854	JN563863
Bothia castanella	28002	—	DQ867119	—
Bothia castanella	28003	DQ867118	DQ867118	—
Bothia castanella	6889	—	DQ867116	—
Bothia castanella	MB03-053	DQ867110	DQ867117	KF030382
Bothia castanella	MB03-067	DQ867115	DQ867115	—
Bothia fujianensis	HKAS82693	KM269194	KM269194	—
Bothia fujianensis	HKAS82694	KM269195	KM269193	—
Buchwaldoboletus lignicola	HKAS 76674	—	KF112350	KF112642
Butyriboletus roseoflavus	HZFA07901	JX290184	JX290184	—
Butyriboletus roseoflavus	HKAS14707	JX290190	JX290190	—
Caloboletus firmus	MB06-060	—	KF030278	KF030368
Calooletus inedulis	MB06-044	—	JQ327013	KF030362
Chalciporus piperatus	HKAS50214	JQ928610	JQ928621	JQ928594

Chalciporus piperatus	MB 04-001	—	DQ534648	—
Chalciporus radiatus	GDGM50080	KP871806	KP871801	—
Chiua virens	HKAS50543	—	KT990550	—
Hemileccinum impolitum	Bim1	—	AF139715	—
Hemileccinum subglabripes	72206	JN563896	KF030303	KF030374
Imleria badia	S-F119691	KJ806970	KJ806971	—
Imleria badia	HKAS53502	KC215204	KC215213	KC215226
Imleria obscurebrunnea	HKAS50477	KC215206	—	KC215233
Imleria obscurebrunnea	HKAS52557	KC215207	KC215220	KC215225
Imleria parva	HKAS55341	KC215202	KC215216	KC215229
Imleria parva	HKAS59437	KC215203	KC215215	KC215228
Imleria subalpina	HKAS56375	KC215209	KC215217	KC215231
Imleria subalpina	HKAS74712	KC215208	KC215218	KC215230
Leccinum monticola	HKAS76669	—	KF112443	KF112592
Paragyrodon sphaerosporus	MB06-066	GU187540	GU187593	—
Paxillus vernalis	AFTOL-ID 715	DQ647827	AY645059	—
Pseudophylloporus boletinoides	**FLAS-F-60407**	**MG845193**	**—**	**—**
Pseudophylloporus boletinoides	**FLAS-F-60413**	**MG845194**	**—**	**—**
Pseudophylloporus boletinoides	**Farid 617**	**MG817716**	**MG817715**	**MG820263**
Phylloboletellus chloephorus	3388	—	DQ534658	—
Phylloporus bellus	HKAS 56763	JQ967239	JQ967196	—
Phylloporus leucomycelinus	HKAS 74678	JQ967249	JQ967206	—
Phylloporus pelletieri	Pp1	DQ534566	AF456818	—
Phylloporus rhodoxanthus	BD374	JN021070	HQ161851	HQ161820
Phylloporus rufescens	HKAS 59722	JQ967263	JQ967220	—
Phylloporus rufescens	HKAS 59723	JQ967264	JQ967221	—
Pulchroboletus roseoalbidus	AMB12757	KJ729486	KJ729499	—

Pulchroboletus rubricitrinus	Farid 335	MF193884	MG026638	—
Retiboletus griseus	Both sn	—	KF030308	KF030373
Rubroboletus dupainii	JAM 0607	—	—	KF030361
Rubroboletus sinicus	HKAS56304	—	KJ605673	KJ619482
Solioccasus polychromus	J. Trappe 15399	JX888459	JQ287643	—
Tylopilus alpinus	HKAS 55438	—	KF112404	KF112538
Tylopilus balloui	REH9467	—	JX889676	—
Tylopilus balloui	Osmundson 1198	—	EU430740	EU434340
Tylopilus felleus	HKAS90203	—	KT990545	KT990913
Uncultured Boletaceae	47C G1 H9	KX899732	—	—
Uncultured Boletaceae	4C G2 C3	KX899785	—	—
Xerocomellus zelleri	REH8724	—	KF030271	KF030366
Xerocomus magniporus	HKAS 59820	JQ678697	JQ678699	—
Xerocomus subtomentosus	K 167686	JQ967281	JQ967238	—
Zangia citrina	HKAS52684	—	HQ326941	—
Zangia roseola	HKAS5266123	JQ928614	JQ928623	Q928595

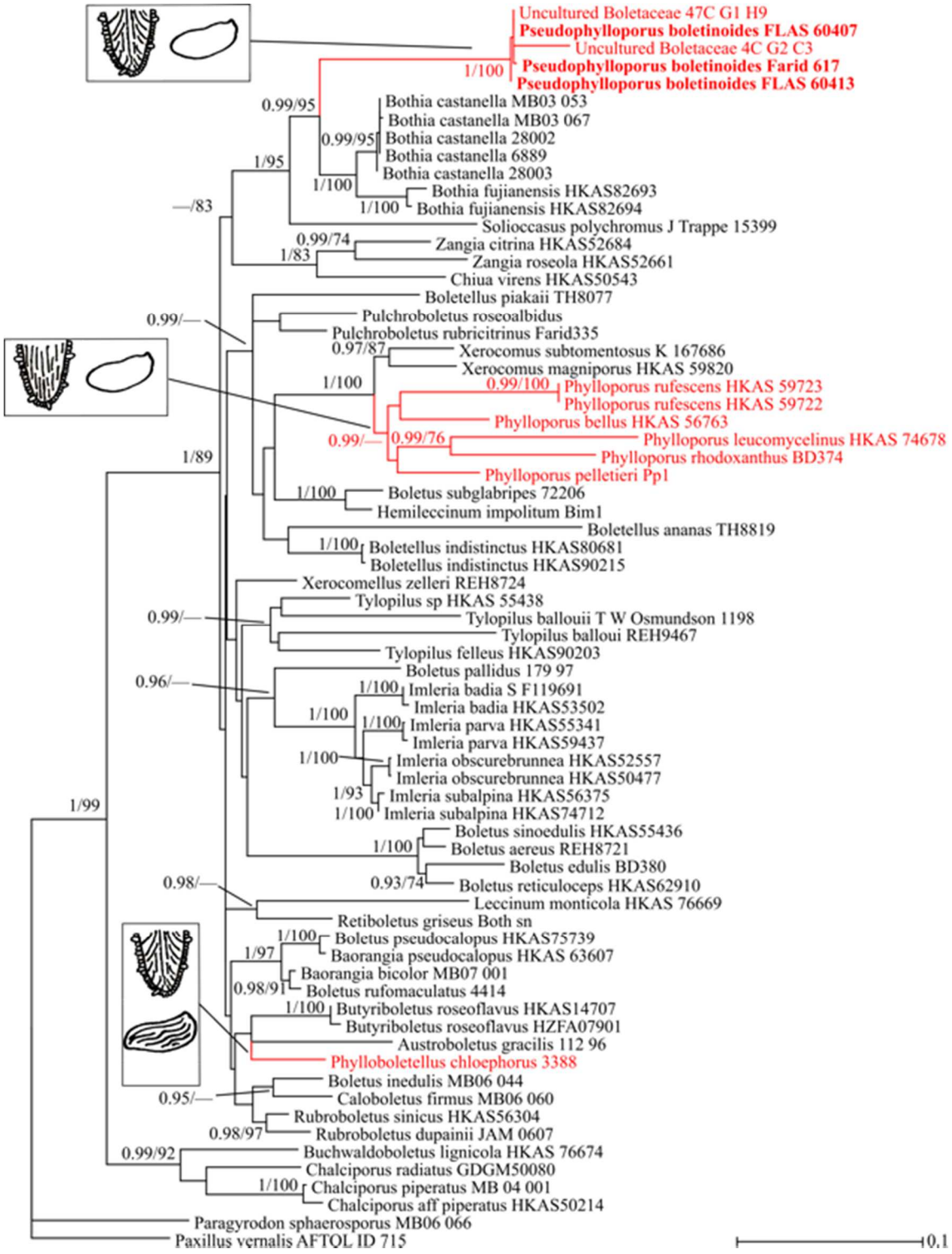

Figure 3.1 – Maximum likelihood tree of the Boletaceae inferred from ITS, LSU, and RPB1 sequences. BPP values 0.95 and above and ML bootstrap values 70% and above are shown adjacent to nodes. The taxon name and the voucher information are shown as the taxon label. Sequences produced in this study are in bold. Lamellate lineages are shown in red. Line drawings of lamellae trama arrangement and spore ornamentation are shown adjacent to lineages.

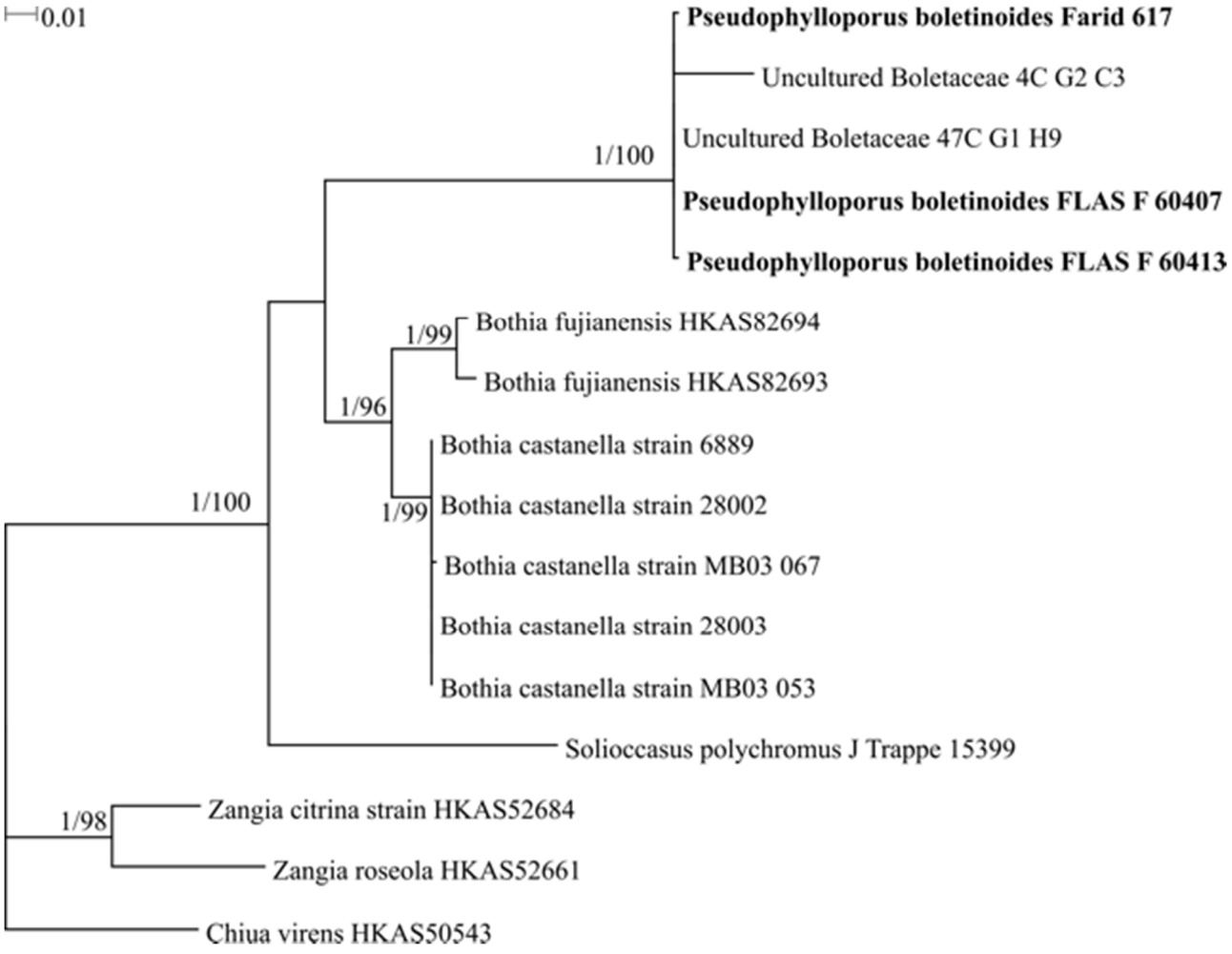

Figure 3.2 – ML tree of the reduced dataset inferred from ITS, LSU, and RPB1 sequences. BPP values exceeding 0.95 and above and ML bootstrap values 70% and above are shown adjacent to nodes. The taxon name and the voucher information are shown as the taxon label. Sequences produced in this study are in bold.

Figure 3.3 – Field photographs of *Pseudophylloporus boletinoides* (Farid 617). Photograph by A. Farid.

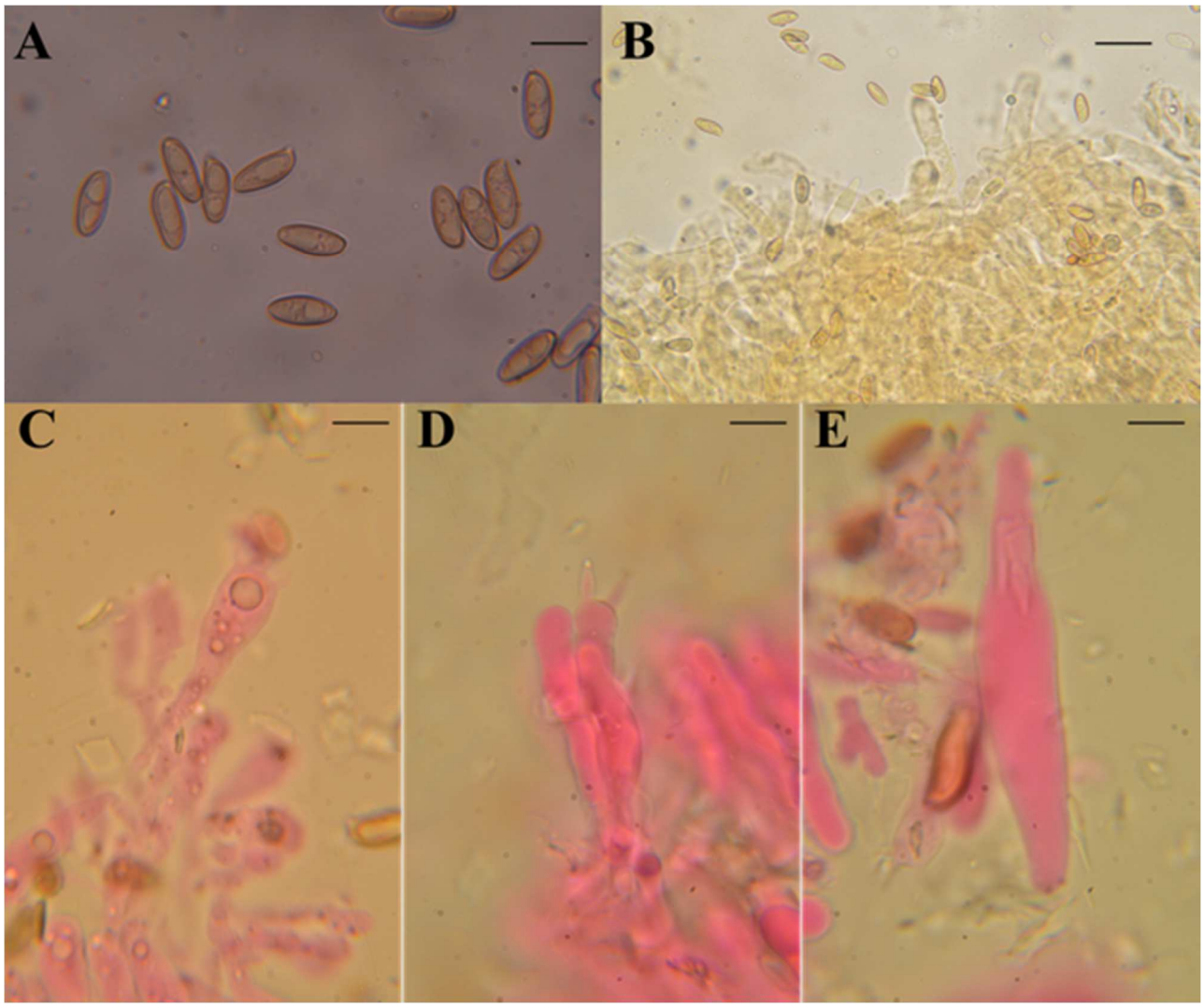

Figure 3.4 – Microscopic features of *Pseudophylloporus boletinoides*. A Spores (Farid 617). B Pileipellis (Farid 617). C–D Basidia and basidioles (C Farid 617, D Baroni BZ-745). E. Pleurocystidia (Baroni BZ-745). Scale bars A, C–E = 10 µm, B = 20 µm. All photographs by A. Farid.

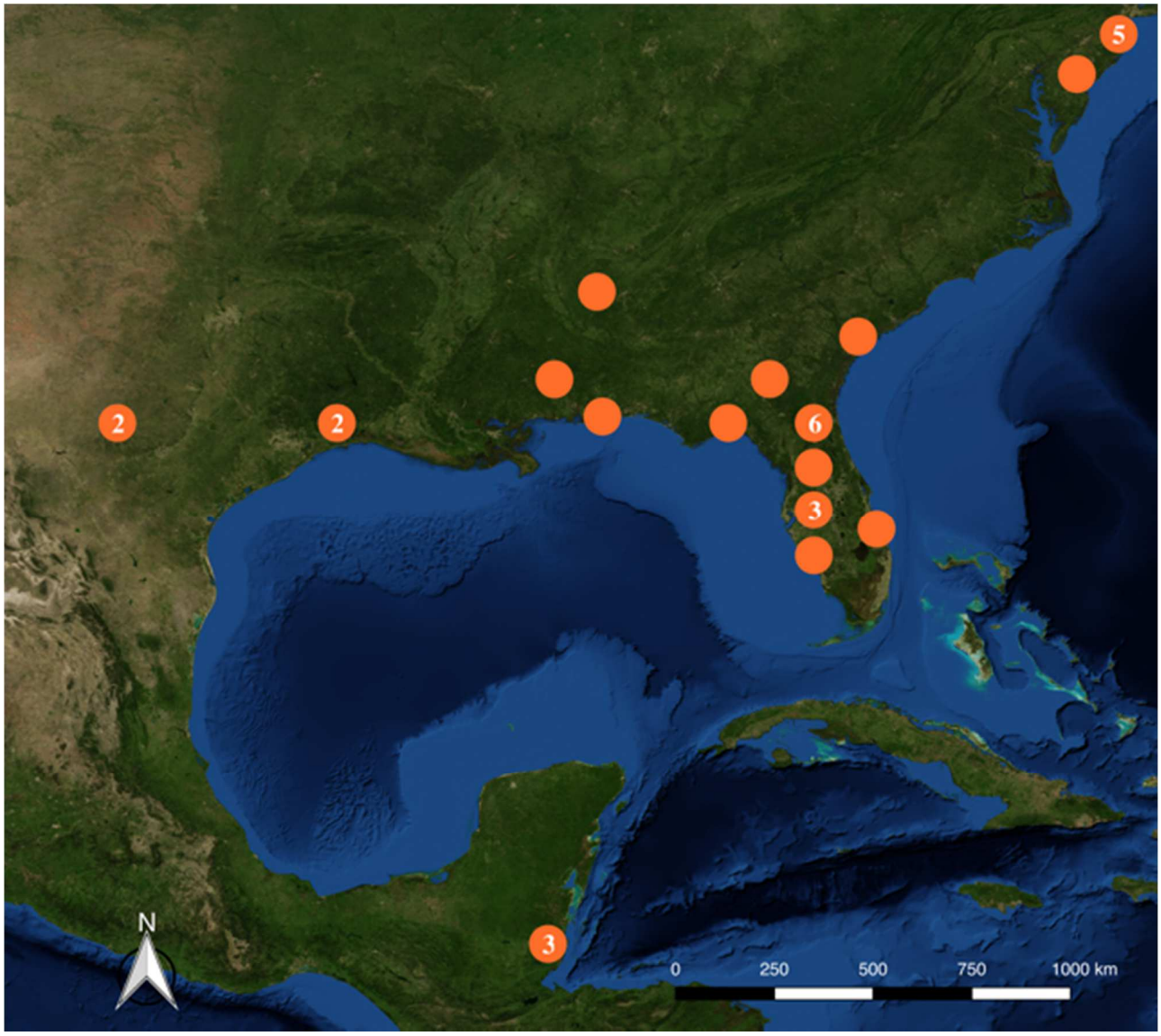

Figure 3.5 – Map generated from mycoportal.org data download (see Appendix) using QGIS version 2.18.2. Aggregated features are shown with numbers indicating the number of collections reported. Coordinate reference system: EPSG:3857.

CHAPTER FOUR: CONCLUSIONS

This study provides the first identified DNA sequences of *Pulchroboletus rubricitrinus* and *Pseudophylloporus boletinoides* to GenBank. As the holotype was in poor condition, an epitype is established for *Pulchroboletus rubricitrinus*. This epitype has molecular data available on GenBank, photographs of fresh specimens, and a more thorough morphological description than the initial holotype. Also, a bolete which forms interesting and unique orange sclerotia was shown to be related to our species. Molecular phylogenetic techniques justified the creation of the genus *Pseudophylloporus* to accommodate *P. boletinoides*, which was previously placed in the genus *Phylloporus*. These phylogenetic analyses also show that lamellae in boletes are a trait which has evolved independently three times from poroid ancestors. Interestingly, *P. boletinoides* groups with secotioid *Solioccasus* and the tubulose *Bothia*, showing a high diversity of fruiting forms which are closely related. An ectomycorrhizal relationship with *Pinus clausa* was also shown using related data from GenBank. These data provide robust phylogenetic analyses for two species which were not previously sequenced, and increase our understanding of bolete evolution.

These data can be used to understand species distribution limits. Additionally, the metadata obtained may be used to understand species habitat preferences, ectomycorrhizal partners, abundance, distribution, which will provide insight into future avenues of research. Studies have utilized these types of data to reconstruct biogeographical histories (Halling et al. 2008, Mathney et al. 2009, Bonito et al. 2013) Molecular techniques utilizing DNA barcoding have been established to quantify the diversity of mycorrhizal fungi to assess plant ecosystem health (Young et al. 2002, Landeweert et al. 2003, Menkis et al. 2005, Kong et al. 2016), but the lack of identified reference sequences limits the capabilities of environmental sequencing.

Fungi have applications in ecological remediation, bioprospecting applications, genomic studies. Despite this broad potential, there is a lack of understanding of fungal diversity to the species level. Species level scientific names are paramount for researchers to communicate fundamental information about species. Research into fungal-derived natural products is moving towards using molecular data for species identification (Raja et al. 2017). These phylogenetic methods may also be useful for industrial applications, such as identifying gene clusters in related species to predict the metabolic activity of an organism (Schmitt & Barker 2009). Dunn et al. (2017) has recently shown the need for phylogenies for genomic analyses. The species richness of mycorrhizal fungi can be used to assess the diversity and health of plant ecosystems; previous studies have shown ectomycorrhizal fungal diversity correlated with plant ecosystem diversity, variability, and productivity (Van der Heiiden *et al*., 1998).

4.1 References

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APPENDICES

APPENDIX A

Specimen data of *Pulchroboletus rubricitrinus* downloaded from Mycoportal.org. Specimens without GPS coordinates were georeferenced using Geocoder (version 1.22.4) with Google set as the geocoding service, and a custom Python script (2.7.10). If locality data could not be obtained, municipality level data were obtained, up to county level. One specimen had only had state-wide level data entered (Texas, D.P. Lewis, 5060), and was excluded from the map. Two specimens (BPI 781720, NCU-F-0002363) were annotated as pieces of Murrill's type collection, and were excluded from the visualization.

United States. Alabama. Baldwin Co., vic. Spanish Fort, Meaher State Park, pine plantation, 22 July 2005, J.L. Mata 1681 (USAM 00121). – Baldwin Co., 21 July 1982, D.P. Lewis 3201 (F C0223076). – Cleburne Co., Cheaha State Park, Cheaha Lake Trail, 3 August 2005, J.L. Mata 1768 (USAM 00207). – Florida. Alachua Co., 27 June 1943, W.A. Murrill F 2380 (FH00489330). – Alachua Co., Gainesville, 26 May 1943, R. Singer 2130 (FH 00489180); ibid., 26 May 1943, R. Singer 2133 (FH 00489331); ibid., 26 May 1943, R. Singer 2135 (FH 00489181); ibid., 28 June 1943, R. Singer 2123a (FH 00489324); ibid., s.d., Murrill (FLAS 15864); ibid., September 1954, W.A. Murrill (BPI 781645); ibid., 936 NW 30th Avenue, 29 July 1982, G. Benny (FLAS 53093); ibid., 1202 NW 16th Avenue, lawn near oaks, 30 July 1982, J. Gibson (FLAS 53107); ibid., at 1401 NW 61st Terr., on the lawn beneath oaks and pines, 11 June 2000, H. Luke (FLAS 57598); ibid., at the entrance of Austin Cary Forest, off of Hwy 24, beneath live oak trees, 14 July 1998, J. Kimbrough (FLAS 56762); ibid., near Fifield Hall, on the lawn beneath *Quercus laurifolia*, 8 July 1998, S. Angels & A. Berry (FLAS 56758); ibid., near Fifield Hall, Hull Rd., beneath live oak tree, 24 July 1997, S. Chandler (FLAS 56570); ibid., Newnan's Lake, edge of pond near Lake, open grass, 8 October 1943, W.A. Murrill (FLAS 19503); ibid., off of NW 4th St. near intersection with NW 10th Avenue, under live oak on median, 29 July 1988, J. Benny (FLAS 55454); ibid., Sugarfoot Hammock, beneath laurel oaks [*Quercus laurifolia*] near open field, 23 July 1969, J. Kimbrough (FLAS 48650). – Highlands Co., 2 September 1942, R. Singer, F181a (FH 00489328). – Hillsborough Co., University of South Florida campus, just N of CCT building, lawn under *Quercus virginiana*, 16 August 2014, A.R. Franck 3473 (USF 275174, USF 275175, USF 275176, USF 275198); ibid., along S side of sidewalk, N of Alumni Drive and S of Richard Beard garage, lawn, beneath *Quercus virginiana*, 10 June 2016, Arian Farid 335 (USF 288420); ibid., along N side of sidewalk, S of Alumni Drive and S of Richard Beard garage, lawn, beneath *Quercus virginiana*, 29 Jun 2017, Arian Farid 575 (USF 293750). – Pinellas Co., St. Petersburg, NW corner of 36th Avenue NE and 1st Street NE, lawn under *Quercus virginiana*, 7 November 2015, A.R. Franck 3970 (USF 282763). – Sarasota Co., Lake Sarasota, 0.2 km S of Bee Ridge Road, 2.2 km E of I-75, under *Quercus laurifolia*, 22 June 2012, A.R. Franck 3036 (USF 273129); ibid., 27 August 2012, A.R. Franck 3114 (USF 273128); ibid., 22 August 2012, A.R. Franck 3112 (USF 273130). – Taylor Co., South side of FL 51, N bank of Steinhatchee River, Steinhatchee, roadside under *Quercus* sp., 27 September 2014, A.R. Franck 3594 (USF 276072).

– Louisiana. St. Tammany Par., Slidell, 8 September 1998, S. Horsch 1780 (F C0223079). – Mississippi. Jackson Co., Gulf Coast Research Lab, scattered to gregarious under *Quercus virginiana*, 25 July 1982, D. Guravich 1523 (MICH 61387). – Long Co., University of Southern Mississippi, Gulf Park Campus, 17 July 1993, W.G. Cibula 1639 (F C0223078). – Texas. Hardin Co., Big Thicket National Preserve, Lance Rosier Unit, 23 July 1983, D.P. Lewis 3544 (F C0223082). – Jefferson Co., Beaumont, Pietsch School, 26 June 1983, D.P. Lewis 3535 (F C0223086). – Orange Co., Vidor, Catholic Church grounds, 28 July 1992, D.P. Lewis 4760 (F C0223084); ibid., near residence, 26 September 1987, D.P. Lewis 4083 (F C0223081). – Tyler Co., Big Thicket National Preserve, Beech Creek Unit, 4 August 1982, D.P. Lewis 3249 (F C0223080). – Tyler Co., Forest Lake Experimental Forest, plot 44, 25 July 1992, D.P. Lewis 4742 (F C0223085).

APPENDIX B

Sepcimen data of *Pseudophylloporus boletinoides* downloaded from Mycoportal.org. Specimens without locality data were excised from the appendix.

United States. Alabama. Baldwin Co., Orange Beach Solitary in sandy area with oaks nearby, 21 July 1982, D. P. Lewis 3196 (F C0235181F). – Mobile Co., Mobile, University of Southern Alabama campus, 20 July 2007, JLM 1954 (USAM-F00393). – Delaware. Sussex Co., Delaware Shore, on white sand dunes and oak-pine forest, 20 August 2012, Martin Livezey (MO 106346). – Florida. Alachua Co., University of Florida (UF) Horticultural Farm near Gainesville, solitary to scattered under Pine, 26 August 1977, W.J. Sundberg VIII-26-77-A-4 (ILLS 00158052). – ibid., UF, Natural Area Teaching Laboratory, on wood, 9 September 2016, Sarah Prentice (MO 253271). – ibid., W side of Newnan's Lake, solitary in deep sandy humus under pines, low hammock, 31 July 1958, H. D. Thiers 4960 (SFSU-F-000741 HOLOTYPE). – ibid., 10 July 2013 Richard and Danielle Kneal (MO 139386). – Citrus Co., Withlacoochee State Forest, Lecanto, growing from the core of a pine [*Pinus*] tree, 23 June 2014, Justin (MO168217). – Glades Co., Fisheating Creek Wildlife Management Area, 1.2 km S of Highlands Co. line, 1.3 km E of C-731, 9 September 2012, Alan R. Franck 3125 (USF 273159). – Hillsborough Co., Violet Cury Nature Preserve, beneath *Quercus* and *Pinus,* N July 2017, Arian Farid 617 (USF 296126). – Polk Co., Green Swamp West Tract, growing on the base of a scorched pine trunk in pine [*Pinus*]/palmetto [*Serenoa repens*] flatwoods, 28 September 2014, Shane Palmer (MO 181528). – Putnam Co., Ordway-Swisher Biological Station, *Pinus* dominated habitat, 23 October 2016, N. Kraisitudomsook NAT-033 (FLAS-F-60407). – ibid., under oaks [*Quercus*] and pines [*Pinus*], 10 October 2016, Matthew E. Smith s.n. (FLAS-F-60413). – Sarasota Co., Englewood, 5 September 2015, (MO239786). – Myakka Valley Ranches, on soil near *Pinus* and *Quercus*, 29 December 1986, R. S. Williams 326 F (F C0224863). – Sarasota, Myakka Valley Ranches, 12 January 1991, R. Singer F-3912 F (F C0235179). – Wakulla Co., Crawfordville, 7 July 2012, Noah Siegel (MO 110081). – Georgia. Brooks Co., 23 June 2017, (MO 279714). – Chatham Co., Pooler, 19 June 2012, Rocky Houghtby (MO 98584). – New Jersey. Burlington Co., Penn State Forest, Oswego Lake, under pine in pine/oak forest, 17 August 1984, R. E. Halling 3811 (NY 15153). – Franklin Parker Preserve, Chatsworth, 3 October 2010, I. G. Safonov (MO 54732). – 0.5 km E of Wharton State Forest, pine [*Pinus*] barrens, 17 July 2011, Walt Sturgeon (MO73726). – Franklin Parker Preserve, Chatsworth, 19 August 2011, I. G. Safonov (MO 74400). – Franklin Parker Preserve, Chatsworth, 18 August 2012, I. G. Safonov (MO 106087). – Texas. Orange Co., Vidor, under mixed pine [*Pinus*] & hardwoods, 17 September 1986, D. P. Lewis 4015 (F C0224867). – ibid., under *Quercus* and *Pinus*, 1987, D. P. Lewis 4090 (F C0235180). – Polk Co., Big Thicket National Preserve, 23 September 2009, Ron Pastorino (MO 25749). – Tyler Co., Kountze Big Thicket National Preserve, Turkey Creek Unit and Turkey Creek Trail, 6 September 1996, Timothy J. Baroni 8172 (CORT 010991). – **Belize.** Cayo District, Douglas da Silva, swamp near British Military Camp, 6 October 2003, T.J. Baroni & R.E. Halling BZ-3224 (CFMR 33694). – Hidden Valley, Lake Lolly Folly, beneath *Pinus caribaea*, 7 January 2002, T.J. Baroni, L. Lacey & B. Ortiz-Santana BZ-745 (CFMR 33444). – Douglas da Silva, Forestry Station, beneath *Pinus caribaea*, 13 October 2002, T.J. Baroni & B. Ortiz-Santana BZ-2108 (CFMR 33601).